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OBSERVATION OF LOOP-TYPE PROMINENCES IN PROJECTION AGAINST THE DISK AT THE TIME OF CERTAIN SOLAR FLARES

By Helen W. Dodson

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Communicated by Robert R. McMath, May 22, 1961

Apparatus and Methods.—For the past five years the spectroheliograph in the 50-foot Solar Tower of the McMath-Hulbert Observatory has been used regularly to obtain records of solar activity in wavelengths on either side, as well as in the centers, of the hydrogen (Hα) and ionized calcium (K) lines.[1] The normal observational procedure in these years has been replacement of the traditional center-of-the-line Hα or K spectroheliogram by a series of 17 spectroheliograms recorded with systematic changes of λ, from 3Å or more on the violet, to 3Å or more on the red side of the center of the respective lines. Each series requires 2 to 4 minutes of observing time. The spectroheliograms made in the wings of the lines have revealed the existence of many abnormalities in the spectrum over localized regions on the solar disk.[1] Such departures from average circumstances can stem from a number of different physical causes.

Many of the conspicuous, large-scale, dark features recorded only, or primarily, on spectroheliograms taken in the wings of Hα lend themselves to interpretation as records of active solar prominences, viewed in projection against the disk. Doppler shifts in the radiation from these phenomena make them essentially "unobservable" on spectroheliograms recorded in the center of Hα. The identification of these dark Hα disk features as active prominences is based upon the ease with which one can recognize in them forms and motions similar to those observed in active prominences seen at the limb of the sun. Study of the λ-sweep spectroheliograms is assisting in the recognition of relationships between those solar phenomena that are usually observed only at the limb of the sun and those that are recorded primarily on the disk.

Observations and identification of certain curvilinear disk features as loop-type prominences in projection: The λ-sweep spectroheliograms have recorded many examples of the familiar phenomenon referred to by flare observers as "active dark flocculi." These can usually be recognized as the disk counterparts of phenomena which if seen at the limb would have been described as ejections, surges, or the development of "activity" in a previously quiescent prominence. In addition, and of especial interest, has been the observation on the disk of features with forms and properties similar to those of the relatively rare loop-type prominences. Up to the present time, we have recorded these features only during the development of certain "important" flares.

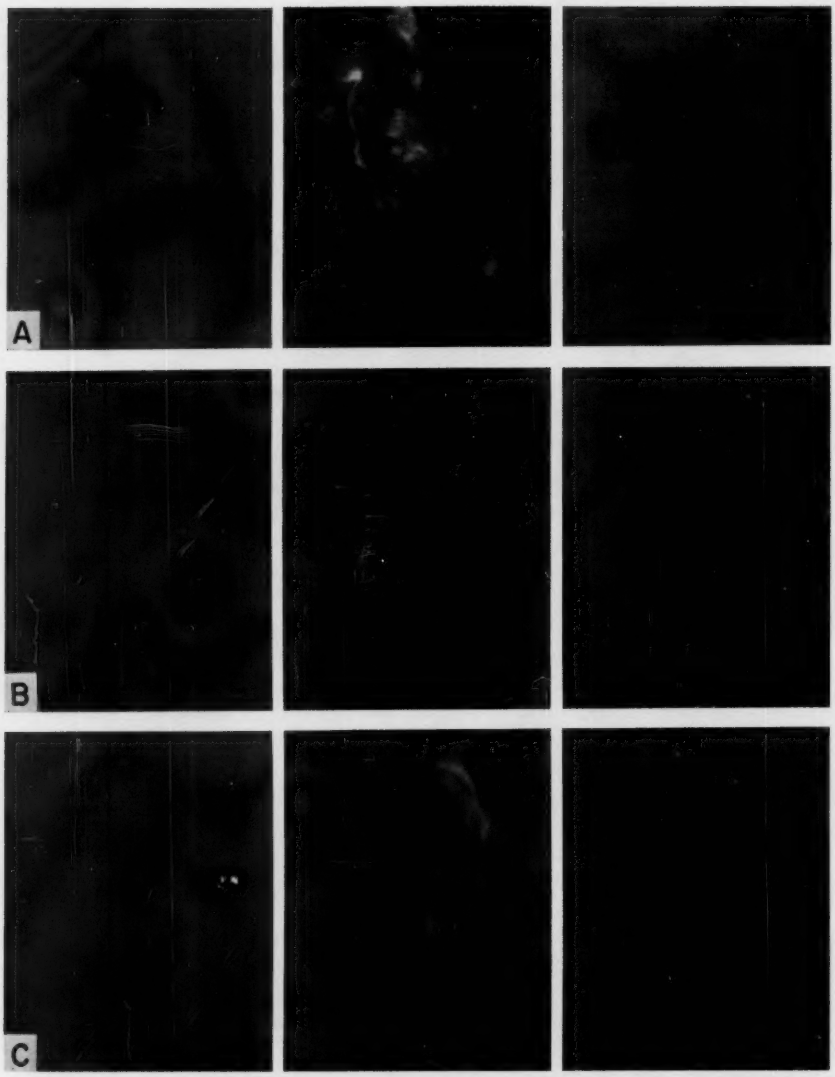

FIG. 1.—Hydrogen spectroheliograms showing "loop-type prominences" projected against the solar disk during the post-maximum phase of three important flares: (A) 1959 July $16^d22^h40^m$ *U.T.*; (B) 1960 March $30^d17^h30^m$ *U.T.*; and (C) 1960 November $12^d16^h20^m$ *U.T.* For each flare, the central picture is the spectroheliogram made with radiation from the center of Hα. For the spectroheliograms in the columns to the left and right, the slit was displaced from the center of Hα by 1.5 Å to the violet and red sides, respectively. Focal length of solar telescope was 40 feet.

The disk features here referred to are curvilinear in shape, comparable in size to loop-type prominences, and are recorded in the red but not in the violet wing of Hα. They were all observed within 40° of the center of the solar disk. In loop-type

prominences observed at the limb of the sun, the apparent direction of motion is "downward," i.e., toward the surface of the sun.[2, 3] The exclusively redward Doppler shifts in the spectrum of the curvilinear features here being considered are consistent with a component of motion directed toward the solar surface. They are thus also consistent with the apparent "downward" motion of loop-type prominences as seen at the limb. The features appear dark against the background of the spectroheliograms made with radiation 0.50Å or more from the center of Hα. The dark, curvilinear features first appear on the λ-sweep spectroheliograms at the time of flare maximum or shortly thereafter. They increase in number, size, and clarity during the declining phase of the Hα flare. They continue throughout the very late stages of the flare or even after the bright Hα flare could be considered as "faded."

Examples of the dark curvilinear structures here described are shown in Figure 1. This figure shows hydrogen spectroheliograms taken during the post-maximum phase of three important flares. The residual bright flare emission can be seen in the pictures in the central column which were made with radiation from the center of Hα. The pictures in the left- and right-hand columns were recorded with radiation from the wings of Hα, 1.5Å from the center of Hα, to the violet and redward sides, respectively. The spectroheliograms made in both the violet and redward wings show the sun spots which were present in the neighborhood of the flares. In addition, the spectroheliograms in the right-hand column (redward wing) show the dark, curvilinear structures here identified as loop-type prominences in projection against the disk.

Disk flares with which the "loop-type prominences" have been photographed: Vast complex systems of these curvilinear structures have been photographed with the 50-ft. Tower Telescope of the McMath-Hulbert Observatory during the post-maximum phase of the following flares:

1959, July 16	21^h14^m–24^h30^m(max. 21^h32^m),	imp. 3+,	16°N, 31°W
1960, March 30	14^h55^m–19^h47^m(max. 15^h40^m),	imp. 2,	12°N, 10°E
1960, June 25	11^h35^m–15^h30^m(max. 12^h15^m),	imp. 2+,	20°N, 05°E
1960, Nov. 12	13^h22^m–18^h30^m(max. 13^h30^m),	imp. 3+,	26°N, 04°W

In addition, on August 22, 1958, a large well-formed, simple loop shows in the redward portion of a λ-sweep made with the flare patrol telescope at 16^h10^m *U.T.* This observation was made during the post-maximum phase of the flare of importance 3 at 18°N, 10°W.

The flares with which the above loop-type structures have been recorded all took place in plages with large sunspots. Furthermore, for each of the flares, part of the bright Hα flare emission occurred directly over the umbra of one or more of the large spots. The flares were all accompanied by strong, long-duration, Type IV radio frequency emission at both centimeter and meter wavelengths. It may be of interest to note that on both July 16, 1959, and November 12, 1960, the 10 cm emission diminished as the system of apparently descending Hα loop-type features developed.

Conclusion.—Comparison of apparent location of "loops" with that of concurrent bright disk Hα flare: Comparison of the position of the bright Hα flare emission with that of the concurrently recorded curvilinear loop-type structures shows

that the two phenomena are not identical. Within the series of spectroheliograms that comprise a λ-sweep, the bright flare emission is best photographed relatively close to the center of Hα while the curvilinear, loop-like features appear most clearly 0.5 to 2.5Å to the redward of Hα. Because of differences in Doppler shift from point to point within the curvilinear features, the entire "loop" is seldom recorded on any one spectroheliogram. On spectroheliograms taken at only 0.5Å from the center of Hα, it is sometimes possible to see in the same picture both the bright Hα flare and the parts of the loop-like features that can be recorded at this wavelength. On these spectroheliograms the two features do not coincide in position.

On spectroheliograms made with successively greater displacements to the redward of Hα, the bright flare ceases to be recorded, and the position, or locus, of the system of dark curvilinear structures changes systematically. With increasing wavelength displacement to the redward of Hα, the locus of the dark curvilinear structures approaches and almost coincides with the position of the bright Hα flare concurrently recorded on the spectroheliograms taken in the center of Hα. The "downward" motion in loop-type prominences at the limb, if observed in projection against the central part of the solar disk, would yield maximum redward Doppler shifts for the "bottoms" or "feet" of the loops. Accordingly, the observations here described suggest that the bright Hα flare emission, during the post-maximum phase of the observed flares was very close in apparent position to the terminal points, or the points of impact with the disk, of a vast and complex system of descending loop-type prominences. For the flares here studied, the main curvilinear parts of the loops did not agree in position with the bright Hα disk flare. These observations indicate that although loop-type prominences are very closely associated with flares, they are not identical with that which appears as the bright "flare" on Hα pictures of the solar disk.

For the flares of July 16, 1959 and November 12, 1960, for the same time intervals in which the complex systems of loop-type prominences were recorded on the λ-sweep spectroheliograms, the feature described by Ellison as a dark "halo" [4] can be discerned on the small-scale, high-contrast Hα flare-patrol pictures secured with the SECASI telescope. The "halo" is a much larger feature than the bright Hα flare and its systems of associated loop-type prominences.

Comparison of apparent positions of loop-type prominences and sunspots: The pattern of the system of loop-type prominences shown on the spectroheliograms is exceedingly complex, and the relationships between true and apparent positions cannot be disentangled unambiguously. In spite of these uncertainties, the spectroheliograms indicate that for the flares studied many of the loop-type prominences had their "feet" in individual spots, and that prominence material apparently descended into the spots. This was especially true for the spots over which the bright Hα flare was apparently superposed. For certain "loops" each of the two branches "descended" into a spot. Some loops apparently had one branch "descending" into a spot and the other branch into a part of the bright Hα flare far from any visible spot.

Up to the present time, the great systems of loop-type prominences have been recorded on λ-sweep spectroheliograms of the solar disk only during the post-maximum phase of certain great flares, flares with Hα emission over the umbrae

of large spots and with concomitant long-duration Type IV radiation at radio frequencies. For other important Hα flares, especially those far from spots (e.g., 1959 July $14^d14^h45^m$), excellent λ-sweep records have been secured, but give no evidence for the occurrence of "loops." Therefore, according to our records to date, the complex systems of loop-type prominences represent an even rarer solar phenomenon than do "important" Hα flares.

[1] McMath, R. R., O. C. Mohler, and H. W. Dodson, these PROCEEDINGS, **46**, 165 (1960).
[2] McMath, R. R., and E. Pettit, *Astrophys. J.*, **85**, 279 (1937) and **88**, 244 (1938).
[3] Sawyer, H. E., and J. Brodie, *Publs. Observatory Univ. Mich.*, **7**, 79 (1938).
[4] Ellison, M. A., S. M. P. McKenna, and J. H. Reid, *Observatory*, **80**, 149 (1960).

THE CLASSIFICATION OF CLUSTERS OF GALAXIES

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Read before the Academy, April 26, 1961

1. The recent publication of the catalogue of clusters of galaxies by George O. Abell[1] is a landmark in extragalactic astronomy; for the first time it is now possible to investigate satisfactorily the general characteristics of rich clusters of galaxies as a class. The present paper gives an application to clusters of galaxies of the system of classification of individual galaxies developed by the writer[2] on the negatives of the National Geographic Society-Palomar Observatory Sky Survey. The introductory parts of this investigation were carried out during periods as a Guest Investigator of the Mount Wilson-Palomar Observatories; the remainder is based on plates obtained with the Yerkes 40-inch refractor, combined with a Meinel reducing camera; the Yerkes plates were taken by Mr. Robert F. Garrison.

The revised classification of the forms of galaxies is a modification of that of Hubble; in effect, one of Hubble's criteria (degree of central concentration of luminosity) has been used exclusively: the resulting classification can be considered a very coarse one; but it does have a physical significance, since it is correlated rather well with spectroscopic evidence concerning the stellar population of the individual galaxies. In particular, two extreme kinds of galaxies can be cleanly separated: (1) galaxies whose light comes principally from hot, blue stars; and (2) galaxies whose light comes principally from yellow giant stars. In addition to these two extremes, galaxies of an intermediate population, consisting of mixtures of (1) and (2) can also be recognized by their form class in the new system of classification.

In class (1) occur a number of highly irregular galaxies and spirals with little central concentration of luminosity; in class (2) are the giant ellipticals, spirals with pronounced nuclei, and certain other galaxies having a high concentration of luminosity in their central regions: in general, the progression in average stellar population from hot stars to the cooler yellow giants changes smoothly with increasing contribution to the total luminosity of the inner regions of each galaxy.

2. Now, when this system of form classification is applied to multiple galaxies and clusters of galaxies, certain common characteristics of the latter emerge. In order to investigate these characteristics further, the rich clusters of galaxies in Abell's nearest groups (distance groups 0 and 1), located north of the equator, were photographed with the 40-inch Yerkes refractor. Since the field of the latter with the Meinel reducing camera is about 20′, the observations apply principally to the nuclear part of each cluster; but each of the 20 clusters in the distance groups observed was examined on the Sky Survey prints to find whether any systematic difference between the galaxies in the inner and outer parts of each clusters existed. No marked differences were found; and it can be taken that the galaxies located in the nuclear region of each cluster are a fairly good index of the nature of the cluster as a whole. For the purposes of the present investigation, this rough comparison is sufficient; but a similar, more precise comparison on the original negatives of the Sky Survey would still be of importance.

3. The conclusions from the reclassification of the visually brightest members of the nearest of Abell's distance groups can be summarized as follows:

(*a*) All of the clusters in the group observed have, for their visually brightest members, galaxies of the centrally-condensed type.

(*b*) The clusters can be divided into two categories according to the types of galaxies encountered one to two magnitudes below the brightest members: (i) those containing appreciable numbers of galaxies of minor central concentration of light; and (ii) those containing few or none of the latter.

(*c*) In terms of the correspondences found between form and stellar population, conclusion (*a*) can be recast as follows: The brightest members of all of the rich clusters in the observed volume tend to have stellar populations of the "evolved" type—yellow giant stars. This would suggest that at some time in the past the physical condition of these rich clusters would have been systematically different from what we observe at the present time.

The question now arises as to how representative of the total observable volume of space the restricted volume appropriate to the present observations can be considered to be. Since no clusters in the observed volume have a richness greater than class 2 on Abell's scale, it has to be concluded that observation of a larger volume, including a number of the richest clusters, would be necessary before the sample can be considered a representative one.

The writer is indebted to Dr. I. S. Bowen and the research committee of the Mount Wilson-Palomar Observatories for permission to use the original negatives of the National Geographic Society-Palomar Observatory Sky Survey. He is also indebted to Dr. W. A. Hiltner and to the latter's contract with the Air Force (Galactic Field Study) for making possible the obtaining of the Yerkes photographs of clusters of galaxies.

The present investigation was supported in part by a contract with the Office of Naval Research.

[1] Abell, George O., *Ap. J.*, Supplement No. 31, 211 (1958).

[2] Morgan, W. W., *Pub. A.S.P.*, **70,** 364 (1958), and *Pub. A.S.P.*, 71, 394 (1959).

*SODIUM-DEPENDENT "TRANSPORT" REACTIONS IN THE CELL NUCLEUS AND THEIR ROLE IN PROTEIN AND NUCLEIC ACID SYNTHESIS**

By V. G. Allfrey, R. Meudt, J. W. Hopkins, and A. E. Mirsky

THE ROCKEFELLER INSTITUTE

Communicated May 10, 1961

It was observed previously that the rate of incorporation of radioactive amino acids into the proteins of isolated cell nuclei increases sharply when sodium ions are added to the incubation medium.[1–3] This sodium requirement for amino acid uptake by the isolated nucleus contrasts with the usual observation that potassium is the specific cofactor needed for the incorporation of amino acids by isolated cytoplasmic systems[4] and for amino acid transport into intact cells.[5]

Why is protein synthesis in the nucleus sensitive to the presence or absence of sodium ions? This question has now been studied using suspensions of isolated calf thymus nuclei. The experiments to be described will show that there is a mechanism, very probably enzymatic, governing and restricting the entry of amino acids into the cell nucleus; this apparent "transport" reaction is sodium-dependent. Thus, the sodium requirement for nuclear protein synthesis reflects the operation of this mechanism for bringing amino acids to the sites of synthesis.

Other synthetic processes in the nucleus may or may not display a similar sodium ion requirement. For example, thymidine-2-C^{14} uptake into DNA and adenosine-8-C^{14} incorporation into RNA both improve when sodium is added to the incubation medium. On the other hand, the utilization of orotic acid for nuclear RNA synthesis does not show a specific sodium ion effect. Here, too, it will be shown that there is a good correlation between the ionic requirements for uptake and for transport of several isotopic precursors of ribo- and deoxyribonucleic acids.

The specific role of sodium in regulating the access of substrates to isolated nuclei suggests its great importance in the regulation of nuclear metabolism *in vivo*. This correlates well with other observations showing that widely diverse cell types have a high intranuclear sodium concentration.

THE EFFECTS OF MONOVALENT CATIONS ON AMINO ACID INCORPORATION INTO NUCLEAR PROTEINS

Isolated calf thymus nuclei can carry out a sustained incorporation of amino acids into their constituent proteins *in vitro*.[6, 1, 2] The isolation of the nuclei has been described previously;[2] it involves homogenization of fresh calf thymus tissue in 0.25 *M* sucrose-0.003 *M* $CaCl_2$ solution, and subsequent isolation of the nuclei by differential centrifugation in the same isotonic medium. It has been shown that thymus nuclei prepared in this way satisfy many morphological and chemical criteria of nuclear purity.[7–10] Moreover, they retain the capacity to synthesize ATP,[11] activate amino acids,[9, 12] and to incorporate a variety of purine and pyrimidine precursors into their nucleic acids.[2, 13–16]

When calf thymus nuclei are suspended in a buffered sucrose medium and incubated at 37° in the presence of C^{14}-labeled amino acids, isotope incorporation into protein occurs; but the amount of radioactivity appearing in the nuclear proteins depends on the nature and concentration of the cations in the incubation

medium. This effect is shown for alanine-1-C^{14} uptake in Figure 1. In this experiment, 1.0 ml aliquots of nuclear suspension were added to 1.0 ml portions of incubation medium containing the radioactive amino acid and increasing concentrations of the chlorides of sodium, potassium, lithium, caesium, or rubidium. (The composition of the medium is given in detail in the legend to Figure 1.) After 60 minutes' incubation at 37°, trichloroacetic acid was added to the nuclear suspensions to bring the final acid concentration to 10 per cent. The nuclei were centrifuged down, discarding the supernates, and the protein was prepared for counting

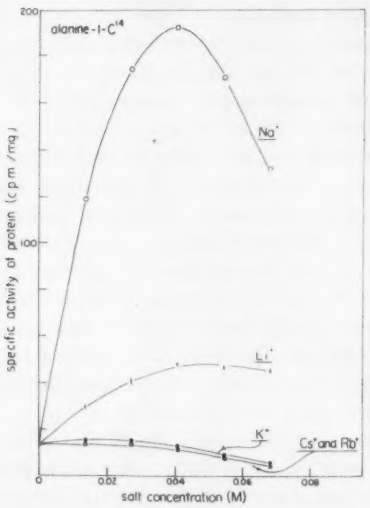

FIG. 1.—Comparative effects of different monovalent cations on the incorporation of alanine-1-C^{14} into the proteins of isolated calf thymus nuclei. The specific activity of the total nuclear protein after 60 minutes' incubation at 37° is plotted against the salt concentration of the medium. The incubation medium is described in detail in the footnote to Table 2. The chlorides of sodium, potassium, lithium, rubidium and caesium were dissolved in the 0.1 *M* glucose solution to produce the final concentrations shown in the figure.

as previously described.[2] Its radioactivity was measured in a thin window, gas-flow G-M counter and the counts corrected for self absorption.[17]

In Figure 1 the specific activity of the nuclear protein (in counts per minute per mg) is plotted against the salt concentration of the medium. It is evident that a medium containing sodium ions provides the optimal environment for C^{14}-alanine uptake into the proteins of the nucleus. The contrast between sodium and potassium-containing media is especially striking. At the concentration optimum (0.04 *M* NaCl under these test conditions) the level of alanine incorporation in sodium-supplemented nuclei is 14 to 18 times greater than that observed at the equivalent concentration of potassium chloride.

Figure 1 also shows the effects obtained with the chlorides of lithium, rubidium and caesium. It is clear that the ions of the heavier alkali metals do not promote amino acid uptake. On the other hand, lithium chloride (at 0.04 *M*) consistently gives alanine uptakes about 4 times higher than those observed when KCl is added.

A few experiments were also carried out to test whether a change in the pH of the incubation medium would alter the nuclear response to sodium ions. In these tests the pH of the medium was varied and the extent of alanine-1-C^{14} incorporation into the proteins of the nucleus was measured as before. A comparison of the uptakes in NaCl and KCl showed that the specific ion requirement is in effect at pH values 6.15, 6.75, and 7.25. As expected, the level of amino acid incorporation fell off rapidly as the pH was lowered from 6.75 to 6.15. The decrease is due, in

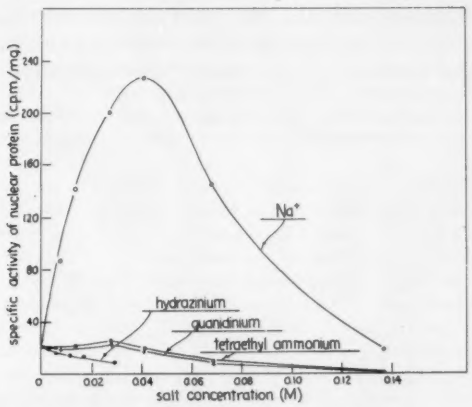

FIG. 2.—Attempts to substitute cationic amines for the sodium requirement of isolated nuclei. The specific activity of the total nuclear protein after 60 minutes' incubation at 37° in the presence of DL-alanine-1-C^{14} is plotted against the salt concentration of the medium. Incubation conditions as in Figure 1.

part, to the diminished activity of the nuclear amino acid activating enzymes[12, 18] but some of the decrease reflects the pH dependence of the nuclear amino acid "transport" reactions (to be discussed below).

Attempts to Substitute for Sodium with Cationic Amines.—A number of quaternary ammonium ions were also tested for possible effects on nuclear amino acid incorporation because Lorente de No[19] had found them to be effective substitutes for sodium ions in maintaining the membrane potential of isolated frog nerve. These tests are summarized in Figure 2, in which the extent of alanine-1-C^{14} incorporation into nuclear protein is plotted against the concentration of electrolyte added to the medium. It is evident that tetraethyl ammonium, hydrazinium, or guanidinium ions cannot replace sodium in this test of function in isolated thymus nuclei. Similar attempts to replace sodium with ammonium ions also led to negative results.

Contrasts in the Sodium Effect Obtained with Different Amino Acids.—The stimulatory effect of sodium ions on amino acid uptake varies for different amino acids. It is particularly large in the case of alanine incorporation, as shown by the high value of the ratio [C^{14}-uptake in NaCl/C^{14}-uptake in KCl], which may reach 14–18 in freshly prepared nuclei under the test conditions described. In earlier studies of glycine-1-C^{14} incorporation, we found the corresponding ratio to be 6–7.[2] The sodium effect is less marked for other amino acids we have studied. As shown in Figure 3, the incorporation of leucine-2-C^{14} and lysine-2-C^{14} is increased by only

about 70 per cent when NaCl replaces KCl in the incubation medium. Similar ratios for C^{14}-uptakes in sodium and potassium-containing media have been observed using tryptophan-2-C^{14} and phenylalanine-2-C^{14}. For valine-1-C^{14} the Na^+/K^+ C^{14}-uptake ratio is 1.85.

Because of the magnitude of the sodium effect on alanine-1-C^{14} incorporation, this amino acid was selected as the material of choice for many of the further studies of the role of sodium ions in protein synthesis by the nucleus.

A Search for Sodium-Dependent Reactions in Nuclear Protein Synthesis.—The incorporation of amino acids into the proteins of the cell nucleus proceeds through a series of reactions[9, 12, 20–22] resembling those observed in cytoplasmic systems.[23] In both cases amino acids are first activated, then transferred to "carrier" ribo-

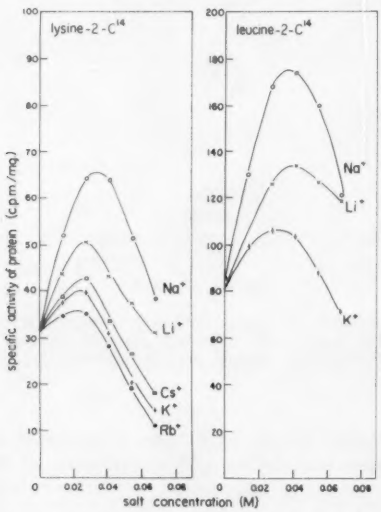

FIG. 3.—Comparative effects of different monovalent cations on the incorporation of lysine-2-C^{14} and leucine-2-C^{14} into the proteins of isolated thymus nuclei. Incubation conditions as in the footnote to Table 2. The isotopic amino acid was added as either DL-lysine-2-C^{14} (0.1 mg, Sp. Act. 0.33 mc per mmole) or DL-leucine-2-C^{14} (0.2 mg, Sp. Act. 0.6 mc per mmole).

nucleic acids, and subsequently incorporated into the proteins of the ribosome fraction by a process requiring GTP.[24, 20] Since the over-all process of amino acid incorporation into the proteins of the nucleus is sodium dependent, it was of interest to see whether any of these individual reactions in the synthetic pathway would also display a sodium ion requirement. The experiments now to be described show that there is no specific sodium ion effect on amino acid activation or transfer to "carrier" RNA in the nucleus.

(a) *A comparison of leucine and valine activation in Na^+ and K^+-containing media:* In the enzymatic activation of amino acids, ATP is cleaved to give inorganic pyrophosphate and an amino acyl-AMP anhydride.[25] The reaction is readily reversible, and, if P^{32}-labeled pyrophosphate is present, radioactive ATP is formed. The incorporation of PP^{32} into ATP can be followed quantitatively by the procedure of Crane and Lipmann.[26] Using this method of assay, it has been shown that isolated calf thymus nuclei contain activating enzymes for at least 15 L-amino acids.[12] Many of these enzymes are precipitated from nuclear extracts when the pH is lowered to 5.2.

In testing for the effect of sodium ions on amino acid activation, the nuclear pH 5 enzyme fraction was prepared in the absence of sodium ions as described earlier[12] and the rate of P^{32}-pyrophosphate-ATP exchange was measured in the presence of added leucine or valine. The activities observed in a sodium-free system and after the addition of 10 μM of NaCl per ml are compared in Table 1. The results show no specific stimulation of the activation reaction by sodium ions.

(b) *Test for Na^+ dependence of amino acid transfer to nuclear RNA:* Since Na^+ was without effect on leucine or valine activation, the amino acid transfer to nuclear "carrier"-RNA was next tested for sodium dependence. In this experiment we measured the transfer of leucine-1-C^{14} to the ribonucleic acid associated with the nuclear pH 5 fraction[9-12] and compared uptakes in sodium and potassium-containing media. The system contained 4 mg of nuclear pH 5 enzymes (4.5 per cent RNA), plus 10 μmoles ATP, 10 μmoles $MgCl_2$, DL-leucine-1-C^{14} (1.09 mc/mmole), and 10 μmoles of either NaCl or KCl in 1.0 ml 0.01 M "tris" buffer at pH 7.6. After 10 minutes of incubation at 37°, the pH 5 fraction was precipitated with 2 per cent perchloric acid and its radioactivity measured. The activity in the Na^+ containing medium was 114 cpm per mg, not significantly different from that observed in the K^+ medium, 105 cpm per mg. In both cases all of the counts could be removed by ribonuclease digestion. Thus there was no specific sodium ion effect on leucine-1-C^{14} transfer to nuclear RNA.

(c) *Leucine incorporation by isolated nuclear ribosomes at low Na^+ concentrations:* We have shown that isolated nuclear ribosomes will incorporate amino acids *in vitro* if they are supplemented with the nuclear amino acid activating enzymes (the nuclear pH 5 fraction), GTP, and an ATP-regenerating system.[20, 21] This incorporation has not yet been studied in a completely sodium-free system. However, some preliminary experiments have been carried out in which up to half of the Na^+ was replaced by an equivalent amount of K^+.[27] The change did not affect the rate of leucine uptake by free ribosomes. The result suggests (though it does not prove) that the synthetic reactions occurring in ribosomes do not specifically require a sodium supplement. This view is supported by the more direct findings presented below in connection with amino acid accumulation in the nucleus prior to protein synthesis.

TABLE 1

TESTS FOR EFFECT OF ADDED SODIUM IONS ON AMINO ACID ACTIVATION* BY NUCLEAR ACTIVATING ENZYMES

Conditions	Enzyme Activity† Without sodium (units)	Enzyme Activity† With 10 μM NaCl (units)
1. Nuclear pH 5 enzymes (1 mg protein) without added amino acids	0.022	0.017
2. Same as 1 + L-leucine	0.065	0.062
3. Same as 1 + L-valine	0.037	0.033

* As measured by ATP-P^{32}-pyrophosphate exchange (12).
† Unit of activity = μmoles of PP exchanged per 10 minutes at 37° (12).

THE ENTRY OR "TRANSPORT" OF AMINO ACIDS INTO THE CELL NUCLEUS

Given the findings that sodium ions can stimulate amino acid incorporation in the intact nucleus without having any apparent effect on protein synthetic reactions in isolated subnuclear components, we next tested the hypothesis that sodium ions might influence the rate at which amino acids enter the free amino acid "pool" within the nucleus. This approach has not only provided an explanation of the sodium effect but it has also revealed the existence of mechanisms, hitherto unknown, for controlling the entry and accumulation of amino acids in the cell nucleus.

A Brief Outline of Procedures.—In studying the penetration of free amino acids into the nuclear "pool," two procedures were used. In both cases, isolated calf thymus nuclei were incubated in the presence of C^{14}-labeled amino acids and, at various time intervals, the nuclei were washed and collected by centrifugation.

Method 1: The nuclear sediment was extracted with cold 2 per cent perchloric acid and the radioactivity of the extract was measured. (In what follows it will be shown that this extract includes the free amino acid "pool" of the nucleus.)

Method 2: In some experiments the "transport" of amino acids was allowed to take place at temperatures too low to permit protein synthesis; the nuclear sediment was then washed with cold acetone and counted directly. Since no amino acid uptake into protein had occurred and all of the counts could be subsequently removed from the nuclei by extraction with dilute acid, the observed C^{14}-activity was taken to represent the radioactive amino acid content of the "pool."

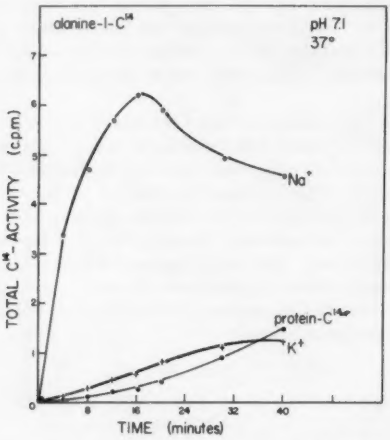

FIG. 4.—Comparative effects of sodium and potassium ions on alanine-1-C^{14} "transport" into isolated nuclei. The total radioactivity present in the nuclear "pool" (in 20 mg of nuclei) is plotted against the time of incubation at 37°. The incubation mixture contained 1.0 ml of nuclear suspension (40 mg nuclei, dry weight), 0.5 ml of 0.1 *M* "tris" buffer in 0.25 *M* sucrose (pH 7.1), 0.4 ml 0.1 *M* glucose containing 10 μmoles $MgCl_2$ and either 8 mg NaCl or 10.2 mg KCl per ml, and 0.1 ml H_2O containing 25 μg DL-alanine-1-C^{14} (Sp. Act. 2 mc per mmole). The C^{14} content of the "pool" was determined at the indicated times by the procedure described in the text (Method 1). The total counts present in the nuclear protein under these conditions are plotted in the lower curve.

Sodium Dependence of Amino Acid "Transport."—The effect of added sodium ions on the entry of amino acids into the nucleus is shown in Figure 4. In these experiments a small, accurately measured amount of DL-alanine-1-C^{14} was added to nuclei suspended in a buffered sucrose medium containing sodium (or potassium) chloride. (The composition of the medium is given in detail in the legend to Figure 4.) The mixture was incubated aerobically with shaking at 37°. One ml aliquots were removed at zero time and at four minute intervals thereafter. These were pipetted directly into centrifuge tubes containing 11.0 ml of cold 0.25 *M* sucrose-0.003 *M* $CaCl_2$. The nuclei were collected by centrifugation at 1–2° (5 minutes at 3000 *g*) and extracted immediately with 4 ml of cold 2 per cent $HClO_4$. This precipitates the nuclear protein and extracts the free amino acids (plus nucleotides and other low-molecular weight, acid-soluble components of the nucleus). The perchloric acid extract was neutralized with KOH, chilled and centrifuged to

remove $KClO_4$, and made up to 5.0 ml with water. Aliquots of the clear solution were taken to dryness in stainless steel cup-planchets and the radioactivity measured as before. After making the correction for the small amount of amino acid adsorbed at zero time, the radioactivity in each extract was plotted as a function of the time of incubation.

In Figure 4 the radioactivity present in the nuclear "pool" after incubation in 0.04 *M* NaCl is compared with that observed at the equivalent concentration of KCl. Alanine transport into the nucleus is clearly a sodium dependent process.

At 37°, the initial rate of alanine entry into the nuclear "pool" is extremely rapid; this makes accurate rate measurements difficult. For this reason, plus the fact that amino acids are not well retained by nuclei at the higher incubation temperatures, most of the subsequent experiments on amino acid penetration were carried out at 10°. At this temperature the transport process is not complicated by the incorporation of radioactive amino acids into the proteins of the nucleus, whereas at 37° an appreciable incorporation of alanine-C^{14} into protein occurs. (This is shown by the lower curve in Fig. 4.)

Evidence that the Radioactivity of the Nuclear "Pool" Occurs as Free C^{14}-Alanine.—In the preceding experiments the alanine content of the nuclear "pool" was determined by measuring the total radioactivity present in the acid extract of washed nuclei after incubation in the presence of DL-alanine-1-C-^{14}. Some tests have been carried out to see whether the radioactivity in the extract occurs as free alanine or whether it exists in some other form. For this purpose the neutralized extract was concentrated and subjected to chromatography on filter paper. The solvent system used was a mixture of secondary butanol, formic acid and water (100 ml sec. butanol:20 ml 88 per cent formic acid. 13.3 ml H_2O); the paper was Whatman No. 1.[28] The system allows a clear separation of an alanine-1-C^{14} spot which could be identified both by staining with ninhydrin and by localization of radioactivity using a windowless strip-scanner and recording ratemeter.

Extracts were prepared from nuclei at zero time and after 15 and 30 minutes' incubation at 10°. The chromatograms of the acid extracts showed no radioactivity in the zero time sample and only one radioactive spot (corresponding in R_f to a C^{14}-alanine standard marker) in the 15 or 30 minute samples. Each of the spots was eluted in water and the eluates taken to dryness in cup-planchets for measurement of radioactivity. The alanine spot in the 15 minute sample had an activity of 125 cpm; after 30 minutes the activity had gone up to 295 cpm. In both cases, 91 per cent of the total counts put on the paper were recovered in the eluates of the alanine spots. This is good evidence that the increase in radioactivity of the nuclear extract with time represents the accumulation of free C^{14}-alanine within the nucleus. This accumulation was not detected in the absence of sodium ions.

Since alanine alone can account for the radioactivity present in the $HClO_4$ extracts, the radioactivity figures can be converted to μg or mμ moles of alanine for calculating "pool" sizes and intranuclear alanine concentrations. The conversion factor (used (1000 cpm = 0.120 μg of L-alanine) was obtained by counting aliquots of the DL-alanine-1-C-^{14} standard solution used in the transport experiments. For reasons given below, further results are expressed in terms of the transport of L-alanine only.

Evidence for the Intranuclear Localization of the Amino Acid "Pool."—In establishing the existence of nuclear amino acid "pools" it is important to minimize or eliminate contamination by intact cells, because whole cells have been shown to concentrate free amino acids to very high levels, often exceeding the concentration of the surrounding medium by several thousandfold.[29]

The elimination of whole cell contamination was carried out in several ways, using both aqueous and nonaqueous methods of nuclear purification. Thymus nuclear suspensions, prepared as described,[2] contain very few intact cells: we have shown that the number of whole thymocytes, as judged by electron microscopy, amounts to about 30 cells per 1000 nuclei.[2,8] This estimate is in accord with cell counts made on stained nuclear preparations under the light microscope.[30]

(a) *Amino acid uptake by nuclei purified in a density gradient:* Because intact cells are less dense than nuclei free of cytoplasm, they can be removed by density gradient centrifugation. In aqueous

systems, we have purified thymus nuclei by centrifugation through 1.95 *M* sucrose or through isotonic (0.25 *M*) sucrose containing Ficoll[31] to raise its density. Using a Ficoll gradient, nuclei were fractionated into dense and light layers, the latter containing the intact cells. A subsequent study of amino acid uptake showed that the dense fractions of purified nuclei still actively incorporated amino acids and that sodium ions were necessary for this uptake. However, in the lighter cell fraction, the rate of C^{14}-incorporation was 7 times higher in a potassium medium than in a sodium buffer of equal molarity. The localization of free alanine-C^{14} in the purified nuclear fraction was also demonstrated by the chromatographic isolation of the amino acid from the $HClO_4$ extract of the nuclei.

(b) *Alanine-1-C^{14} retention by nuclei purified in nonaqueous media:* Nonaqueous methods of fractionation have also been applied to remove the few remaining cells from calf thymus nuclear suspensions. In these experiments the purification was carried out after alanine "transport" had occurred at 10° (a temperature too low to permit nuclear protein synthesis). Following incubation for 30 minutes in the usual buffered sucrose medium (with either NaCl or KCl present), the nuclei were washed and collected by centrifugation. After dehydration in cold acetone, the nuclear sediment was resuspended in a cyclohexane-CCl_4 mixture of density 1.40 and centrifuged at 3000 *g* for 20 minutes. The supernatant fraction, containing light nuclei and some cells, was decanted and dried (Fraction S). The sediment was resuspended in cyclohexane-CCl_4 of specific gravity 1.43; a medium sufficiently dense to float the nuclei away from a small amount of dark, heavy debris. The purified nuclei were then collected by centrifugation at Sp. Gr. 1.40 (Fraction R).

The two nuclear fractions (S and R) were plated on filter paper planchets and their radioactivity determined as before.[2] The specific activity of the pure nuclei (Fraction R) was 483 cpm/mg after alanine-1-C^{14} transport in NaCl; the light nuclear fraction S (containing the cells) had 443 cpm/mg. The corresponding figures in the potassium experiment were 75 and 70 cpm/mg respectively. All of these counts could be removed from the purified nuclei by extraction with cold 2 per cent perchloric acid and identified as alanine-C^{14}.

The occurrence of free amino acids in isolated thymus nuclei was first shown by Kay[32] who prepared the nuclei directly from lyophilized tissue by the Behrens' procedure.[33, 34] This is very strong evidence for the occurrence of nuclear amino acid "pools" *in vivo* and gives further support to the view that the accumulation of free amino acids by thymus nuclei *in vitro*, as reported here, can be considered a normal physiological activity.

(c) *Localization of alanine-C^{14} by autoradiography of nuclei:* As a further check on the intranuclear localization of the isotopic alanine, the nuclear sediments in the preceding experiment (Fractions R) were spread on microscope slides and covered with dry stripping-film (Kodak AR-10) for autoradiography. After two weeks in darkness, the slides were developed and the nuclei stained with Giemsa's stain.[35] Microscopic examination showed grains above many of the nuclei in the sodium experiments and no appreciable radioactivity in the nuclei of the potassium series. There was no evidence for exclusive isotope localization in intact cells.

Thus the results of fractionation and autoradiographic experiments substantiate the view that free amino acids are being accumulated in an intranuclear "pool," and that this process is sodium-ion dependent.

The Influence of Concentration on Amino Acid "Transport."—The rate of amino acid entry into the nuclear "pool" depends on the amino acid concentration of the medium. This relationship is shown for alanine-1-C^{14} transport in Figure 5, in which the rate of alanine entry (μg transported in 15 minutes) is plotted against the alanine concentration of the incubation medium. In all cases the nuclear concentration was fixed at 20 mg (dry weight) per ml of incubation mixture.

The shape of the curve recalls the familiar effect of increasing substrate concentration on the velocity of an enzymatic reaction. At first the rate of alanine entry into the nucleus increases as the concentration of external alanine is raised. With higher concentrations the rate of transport reaches a rather abrupt plateau and thereafter there is no further accumulation of amino acid by the nuclear "pool." (The kinetics of alanine entry into the nucleus are not simply expressed by the

Michaelis-Menten equation,[36] since a Lineweaver-Burk plot[37] of $1/v$ against $1/S$ (where v is the rate of alanine transport, and S is the concentration of alanine in the medium) deviates appreciably from linearity at the higher substrate concentrations. This, of course, is not surprising in view of the complexity of the intact nucleus as an assay system.)

The resemblance between nuclear amino acid transport and an enzymatic reaction is not limited to the similar effects of increasing substrate concentration on the two processes. It will be shown below that the transport reaction also has a high degree of specificity for the naturally occurring L-isomers of the amino acids, in addition to having well-defined pH and temperature optima.

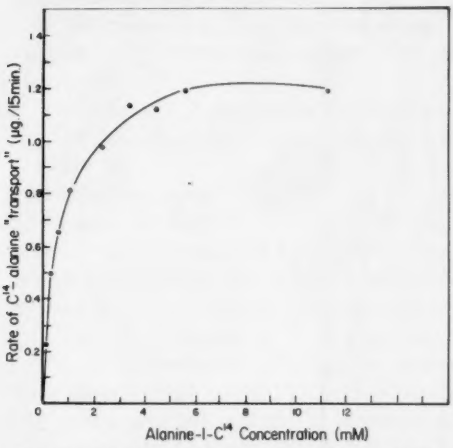

FIG. 5.—The effect of increasing the alanine-1-C^{14} concentration of the external medium on the initial rate of C^{14}-alanine entry into the nuclear "pool." The rate of transport is expressed as the number of micrograms of alanine transported into 20 mg nuclei during the first 15 minutes of incubation. This is plotted against the alanine concentration of the medium. The incubation mixture contained 1.0 ml of nuclear suspension, 0.5 ml of 0.1 M sodium phosphate buffer in 0.25 M sucrose (pH 6.75), 0.4 ml of 0.1 M glucose containing 3.75 mg NaCl and 10 µmoles $MgCl_2$, and 0.1 ml H_2O containing the indicated amounts of DL-alanine-1-C^{14}. The C^{14}-content of the "pool" after 15 minutes at 10° was determined as described in the text (Method 2).

Relative Alanine Concentrations in Nuclei and Surrounding Media.—By comparing the radioactivity present in nuclear extracts with that of the incubation medium after transport has occurred, estimates can be made of the relative alanine-C^{14} concentrations within and outside the nucleus. Several such comparisons have been made, both in sodium and in potassium-containing media. The procedure was as follows: incubations were carried out at 10° in the buffered sucrose medium described in the legend to Figure 4. Under these conditions the alanine content of the nucleus reaches a maximum at about 60 minutes. At that time the nuclei were washed with cold 0.25 M sucrose solution and collected by centrifugation in graduated tubes. The volume of the packed nuclear sediment was measured and the amino acids were extracted with cold 2 per cent perchloric acid. Aliquots of the extract were neutralized with KOH and taken to dryness for determination of their C^{14}-activity. Appropriate dilutions of the incubation medium were counted in the same way. Using the factor relating radioactivity to alanine-1-C^{14} content (1,000 cpm = 0.120 µgm alanine), the alanine concentration of the nuclear sediment was calculated and compared with that of the incubation medium.

In a typical experiment the nuclei contained 3.7 µgm of L-alanine per ml (of total nuclear volume) as compared with 3.9 µgm/ml in the medium. In another test the intranuclear alanine concentration was 4.2 µgm/ml while that of the medium was 4.5 µgm/ml. Thus, on the basis of total nuclear volume, the intranuclear concentration of free amino acid closely approximates that of the medium but does not exceed it. However, if the amino acid "pool" of the nucleus is sequestered to

only a portion of its volume (as seems likely in view of the space occupied by chromosomes, nucleoli, and nucleolar ribosomes), then the alanine concentration within the "pool" exceeds that of the surrounding medium. In either case, the results cannot be explained in terms of simple diffusion, since the amino acid fails to enter the "pool" in the absence of sodium ions. Amino acid entry into the nucleus appears

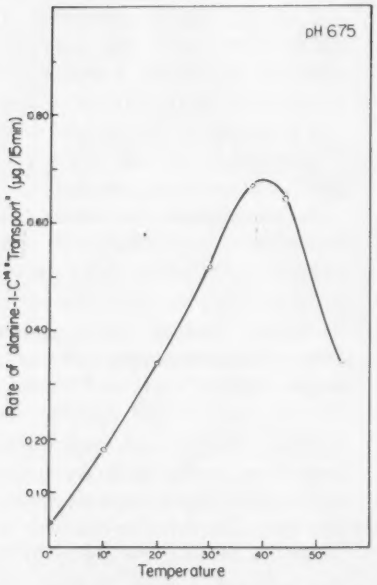

FIG. 6.—Temperature dependence of the alanine transport reaction. The rate of alanine-1-C^{14} entry into the nuclear "pool" is plotted against the incubation temperature. The incubation conditions are given in the legend to Figure 5, except that in all cases the nuclei received 25 μg of DL-alanine-1-C^{14} (Sp. Act. 2 mc per mmole).

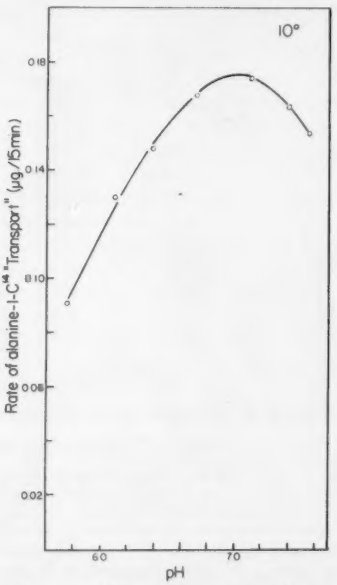

FIG. 7.—Effect of varying pH on alanine-1-C^{14} transport into isolated thymus nuclei. The rate of alanine entry into the nuclear "pool" is plotted against the pH of the incubation medium. In these experiments the pH was varied by changing the ratio of K_2HPO_4 to KH_2PO_4 in the buffer, keeping the total phosphate concentration at 0.1 *M*. The incubation medium is described in Table 2. Incubation temperature = 10°.

to involve a specific "transport" or "gate" mechanism which is sodium dependent. Evidence will now be presented for the enzymatic nature of this process.

Enzyme-like Properties of the Amino Acid Transport Mechanism.—(a) *Temperature dependence:* The relationship between the rate of alanine transport into the nucleus and the temperature of incubation is shown in Figure 6. In these experiments, aliquots of a nuclear suspension containing the optimal concentration of sodium ions were incubated at 0°, 10°, 20°, 30°, 38°, 44°, and 55°. The radioactivity entering the nuclear "pool" was measured at intervals up to 30 minutes and the rate of transport was calculated in terms of the amount (in μgm) of C^{14}-alanine entering the "pool" in the first 15 minutes of incubation. This initial rate is plotted against temperature in Figure 6.

The apparent optimum for the reaction is in the physiological temperature range. There is no transport in nuclei heated to 60° for 10 minutes and very little uptake into nuclei at 0°. At the temperature optimum (38°) the transport kinetics are complicated by the utilization of the free amino acids for nuclear protein synthesis and by the inability of the nuclei to retain peak amino acid levels for more than 15–20 minutes. (At higher temperatures (e.g., 55°) the retention of alanine is very poor and the radioactivity of the "pool" declines after only 10 minutes' incubation. For this reason it is likely that the transport rates measured at elevated temperatures require correction for the loss of amino acid attending changes in the state of the nuclei.)

A comparison of the rates of alanine transport at 10° and at 20° (0.181 and 0.341 μgm of L-alanine/15 minutes, respectively) shows that the velocity has doubled for this 10° rise in temperature. Such a temperature coefficient ($Q_{10} = 2$) indicates that amino acid entry into the nucleus probably involves a chemical reaction and cannot be explained in terms of simple diffusion, since the temperature coefficient of the latter process is relatively small. Moreover, the fact that the rate of alanine accumulation by nuclei increases with increasing temperature (between 0° and 38°) argues against a simple adsorption mechanism, since adsorption would be expected to decrease as the temperature of the adsorbing surface is raised.

(b) *pH dependence:* The rate of alanine transport into isolated nuclei varies with the pH of the incubation medium as shown in Figure 7. In these experiments the system was buffered at the indicated pH values by using a 0.025 *M* phosphate buffer and varying the ratio of K_2HPO_4 to KH_2PO_4. The sodium concentration was fixed at the previously determined optimum for alanine transport at pH 6.75 (0.04 *M* NaCl). At each pH value the radioactivity of the nuclear "pool" was measured at intervals up to 30 minutes at 10°. The rate of uptake is expressed in terms of the amount of L-alanine transported into 20 mg of nuclei (dry weight) in the first 15 minutes of incubation. This rate is plotted against pH in Figure 7.

It is clear that there is a well-defined optimum at neutrality, and that the rate of transport falls off rapidly at pH values below 6.8. (This decline in alanine transport at low pH values is one of the major reasons for the low C^{14}-incorporations observed in the earlier studies of nuclear protein synthesis at pH 6.15.)

(c) *Specificity of the transport reaction for the L-isomer of the amino acids:* In the preceding experiments on C^{14}-alanine transport into the nucleus, the labeled amino acid was added as DL-alanine-1-C^{14}; that is, the racemic mixture of the L- and D-optical isomers of the acid prepared by chemical synthesis. It was not known whether both components of this racemic mixture could enter the nuclear "pool" or whether the transport reaction was specific for the naturally occurring L-form of the amino acid. To test this point we made use of competition experiments in which a large excess of nonradioactive L-alanine or D-alanine was added to the nuclear suspension before the addition of DL-alanine-1-C^{14}. The results of these experiments are summarized in Table 2. As expected, unlabeled L-alanine competed with the radioactive alanine for transport into the nucleus and, as a result, the radioactivity of the nuclear "pool" was diminished. However, the D-isomer of the unlabeled amino acid did not produce a corresponding decrease in the C^{14}-alanine content of the nuclear "pool," and it can be concluded that unlabeled D-alanine did not compete with C^{14}-alanine for transport. It follows that in the

racemic mixture, DL-alanine-1-C^{14}, the D-isomer is not actively taken up by the nucleus, and that the radioactivity observed in the nuclear "pool" represents the specific accumulation of the L-form of the amino acid.

Similar evidence that the transport mechanism specifically selects the naturally occurring isomer of the amino acid has been obtained with valine-1-C^{14} and leucine-1-C^{14} (see below). It is also of interest in this connection that later stages in nuclear protein synthesis, beginning with amino acid activation,[12] can also discriminate against the D-amino acid isomers.

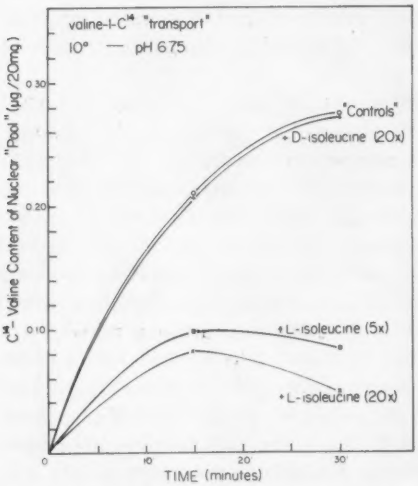

FIG. 8.—Effect of added D- and L-isoleucine on the transport of valine-1-C^{14} into isolated nuclei. The C^{14}-valine content of the nuclear "pool" is plotted as a function of the time of incubation at 10°. The incubation mixture contained 1.0 ml of nuclear suspension, 0.5 ml of potassium phosphate buffer in 0.25 *M* sucrose (pH 6.75), 0.4 ml 0.1 *M* glucose containing 10 μmoles $MgCl_2$ and 12 mg NaCl per ml, and 0.1 ml H_2O containing 50 μg. DL-valine-1-C^{14} (Sp. Act. 1.8 mc per mmole). Unlabelled D- or L-isoleucine was dissolved in the buffer to give a concentration 5 or 20 times greater than that of the radioactive valine.

Competition between Similar Amino Acids for Transport into the Nucleus.—The transport of valine-1-C^{14} into the nuclear amino acid "pool" is progressively diminished as increasing amounts of its structural analogue, L-isoleucine, are added to the incubation medium. The effect is illustrated in Figure 8, in which the time course of valine uptake in the absence of isoleucine is compared with that observed in the presence of a 5- or 20-fold excess of unlabeled isoleucine. The figure also shows that only L-isoleucine competes with valine for transport, since D-isoleucine does not affect the transport reaction under these conditions.

Similar competitive effects are observed between alanine-1-C^{14} and unlabeled glycine (see Table 3) but the addition of dissimilar amino acids such as isoleucine, lysine, or glutamic acid has little or no effect on alanine-C^{14} transport into the nucleus.

The results indicate that specific receptor sites are concerned with alanine or valine transport. These sites can readily discriminate between the L and D isomers of the appropriate amino acids, but close structural analogues such as L-isoleucine or glycine can competitively occupy the sites involved in valine or alanine transport, respectively. Such steric specificity is characteristic of enzymatic processes, and it may well be that the transport of different amino acids into the nucleus is catalyzed by specific enzymes of the "permease" type.[38]

Amino Acid Accumulation as a Prelude to Protein Synthesis.—The ability of the nucleus to accumulate and retain amino acids has made it possible to test whether sodium ions must always be present in the medium in order for nuclear protein synthesis to occur. In these experiments the transport of alanine-1-C^{14} was allowed to proceed for 30 minutes at 10°. (No C^{14}-incorporation into protein occurs under these conditions.) The nuclei were washed and resuspended in 0.25 *M*

TABLE 2

EFFECTS OF UNLABELED D-ALANINE AND L-ALANINE ON NUCLEAR TRANSPORT OF DL-ALANINE-1-C^{14}

Conditions*	Rate of C^{14}-Alanine Transport† "Counts" per 15 min	μgm per 15 min	Rate relative to "control" (per cent)
"Control" nuclei	9320	1.12	100
Nuclei + 250 μgm D-alanine	9105	1.09	97.3
Nuclei + 500 μgm D-alanine	8445	1.01	90.2
Nuclei + 250 μgm L-alanine	3725	0.447	39.9
Nuclei + 500 μgm L-alanine	3180	0.382	34.1

* The incubation mixture contained 1.0 ml of nuclear suspension (40 mg nuclei, dry weight), 0.5 ml of 0.1 *M* potassium phosphate buffer in 0.25 *M* sucrose (pH 6.7), 0.4 ml of 0.1 *M* glucose containing 12 mg NaCl per ml and 10 μmoles $MgCl_2$, and 0.1 ml H_2O containing 50 μgm of DL-alanine-1-C^{14} (specific activity 2 mc per mmole). The D- and L-alanine supplements were added in 0.1 ml H_2O prior to addition of the isotopic alanine. Incubation temperature = 37°.

† Rate expressed as μgm of C^{14}-alanine transported into 20 mg nuclei in the first 15 minutes of incubation.

sucrose-0.003 *M* $CaCl_2$. Half of the nuclear suspension was transferred to a medium containing the optimal concentration of Na^+; the other half was added to an all-potassium system. Both suspensions were then incubated aerobically at 37°. Since no more C^{14}-alanine was added, only previously accumulated alanine could be used for protein synthesis.

A comparison of the time courses of C^{14}-incorporation in the Na^+ and K^+ systems is shown in Figure 9*B*. It is evident that once alanine has entered the "pool," uptake into protein can now take place in the absence of added sodium ions. Although the rate of C^{14}-uptake in an all-potassium system lags behind that of the sodium supplemented nuclei, it is still of the same order of magnitude. This contrasts with the results obtained when nuclei are not allowed to accumulate alanine first but are placed directly into either Na^+ or K^+ media with the isotopic amino acid (Figure 9*A*). Here the rate of uptake at 37° is 18 times greater in the sodium- than in the potassium-medium.

The experiment shows that protein synthesis in the nucleus is sodium-dependent largely because sodium ions are needed to get amino acids to the site of synthesis. Once transport has occurred, the continued presence of sodium ions in the medium is not necessary for incorporation. This finding is in accord with the earlier observations that amino acid activation and transfer to "carrier-RNA" are not sodium-dependent reactions.

Retention of Amino Acids in the Nuclear "Pool."—It is evident from the results

TABLE 3

TESTS FOR COMPETITIVE EFFECTS OF OTHER AMINO ACIDS ON ALANINE-1-C^{14} TRANSPORT INTO ISOLATED NUCLEI

Conditions*	C^{14}-alanine Content (μ gm) of the "Pool"† at 15 minutes	30 minutes
"Control" nuclei	0.178	0.348
Nuclei + glycine	0.148	0.209
Nuclei + D-isoleucine	0.187	0.343
Nuclei + L-isoleucine	0.167	0.287
Nuclei + D-glutamic acid	0.176	0.341
Nuclei + L-glutamic acid	0.174	0.324
Nuclei + D-lysine·HCl	0.171	0.349
Nuclei + L-lysine·HCl	0.171	0.351

* The incubation mixture contained 1.0 ml of nuclear suspension (40 mg nuclei), 0.5 ml of 0.1 *M* sodium phosphate buffer in 0.25 *M* sucrose (pH 6.7), 0.4 ml of 0.1 *M* glucose containing 3.75 mg NaCl per ml and 10 μmoles $MgCl_2$, and 0.1 ml H_2O containing 50 μgm (0.56 μmole) of DL-alanine-1-C^{14} (Sp. Act. 2 mc per mmole). The indicated unlabeled amino acids were dissolved in the buffer solution in quantities sufficient to exceed the L-alanine concentration by 10-fold (2.8 μmoles per flask). Incubation temperature = 10°.

† "Pool" size expressed as μgm per 20 mg nuclei (dry weight).

summarized in Figure 9*B* that nuclei can retain amino acids accumulated at low temperatures and utilize them later for protein synthesis at 37°. A number of experiments have been carried out to measure the stability of the nuclear amino acid "pool," but because the results vary from one preparation of nuclei to another, the findings will not be presented here in detail. We know now that isolated nuclei cannot retain amino acids for extended periods. At 10°, the average nuclear "pool"

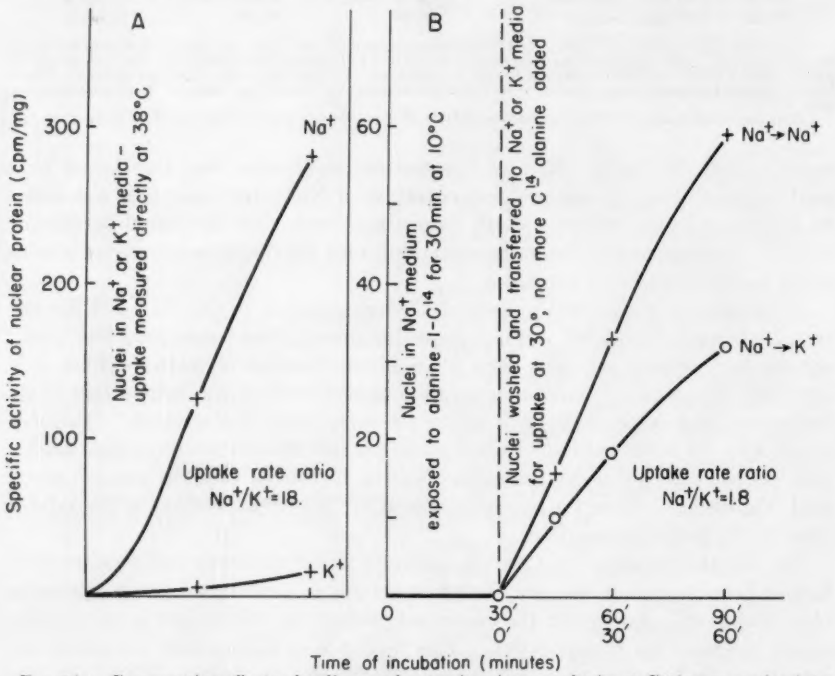

FIG. 9A.—Comparative effects of sodium and potassium ions on alanine-1-C^{14} incorporation into the proteins of nuclei which had not previously accumulated the amino acid in the nuclear "pool." The specific activity of the nuclear protein is plotted against the time of incubation at 37°.

FIG. 9B.—Amino acid incorporation from a prelabeled nuclear "pool." Nuclei were allowed to accumulate alanine-1-C^{14} for 30 minutes at 10° in the presence of sodium ions. The nuclei were centrifuged and washed, and then resuspended in media containing either Na^+ or K^+. No additional C^{14}-alanine was added. The extent of subsequent C^{14}-alanine incorporation into nuclear protein at 37° is plotted against the time of incubation.

is stable for about an hour. At higher temperatures, amino acids enter the "pool" more rapidly but are not as well retained. Thus, at 37° (as shown in Fig. 4) the alanine content of the "pool" begins to decline after only 15–20 minutes.

Tests for Amino Acid Exchange.—It is a matter of some interest to know whether the entry of amino acids into the nuclear "pool" is a reversible process. This has been investigated by testing whether C^{14}-alanine can be displaced from the nucleus by adding C^{12}-alanine to the surrounding medium. The results of such a test are shown in Figure 10, in which the C^{14}-activity of the nuclear "pool" (prelabeled with alanine-1-C^{14} at 10°) is plotted against the time of incubation in the presence of a 200-fold excess of unlabeled alanine. Under these conditions about a third of the

original counts were lost after 30 minutes' incubation with L-alanine. Half of this loss is due to the irreversible leakage of C^{14}-alanine from the "pool," as seen in "control" nuclei and in nuclei receiving a D-alanine supplement. Therefore only about 17 per cent of the C^{14}-alanine "pool" was replaced by C^{12}-alanine in this 30-minute interval. The exchange has been observed at 0° and at 10°, but it is difficult to detect at higher temperatures because of the shortened life of the "pool."

These findings in thymus nuclei are reminiscent of the very interesting work of

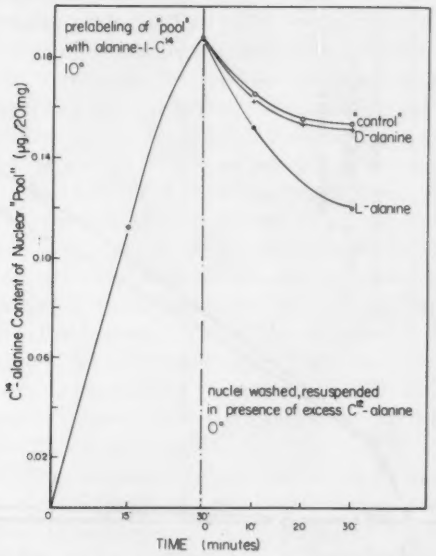

FIG. 10.—Loss by "exchange" of C^{14}-alanine previously taken up into the nuclear amino acid "pool." In this experiment the nuclei were allowed to accumulate alanine-1-C^{14} for 30 minutes at 10° (in the medium described in Table 3). They were then centrifuged down and washed. The nuclear sediment was resuspended in the presence of 5.0 mg of unlabeled D- or L-alanine. (This is 200 times the amount of C^{14}-L-alanine originally added.) The C^{14}-alanine content of the nuclear "pool" is plotted as a function of the time of subsequent incubation at 0°.

Britten *et al.* on proline exchange in bacterial cells[39] and it may well be that amino acid accumulation and exchange involve a similar transport mechanism in these diverse systems.

Failure of the Nuclei to Retain Sodium Ions.—Although isolated thymus nuclei can retain free amino acids, they cannot retain the sodium ions necessary for amino acid transport. This is shown by the preincubation experiments summarized in Figure 11. In one series of tests, aliquots of a nuclear suspension were added to a medium containing sodium ions and shaken at 10° for ten minutes. (No C^{14}-alanine was present during this preincubation period.) The nuclei were then centrifuged down, washed with cold 0.25 *M* sucrose-0.003 *M* $CaCl_2$, and resuspended in sucrose-$CaCl_2$. The suspension of sodium treated nuclei was then divided into two parts, one of which was added to a medium containing 0.04 *M* Na^+, the other to an all K^+ system. A small amount of alanine-1-C^{14} was added to each sample and the radioactivity entering the nuclear "pool" was measured as previously described (Method 1).

Comparison of the transport curves ($Na^+ \rightarrow Na^+$) and ($Na^+ \rightarrow K^+$) shows that a prior exposure to sodium ions does not enable nuclei to accumulate alanine later in an all-potassium medium. The sodium necessary for the transport reaction is not retained, and must be present in the medium at the time C^{14}-alanine is added.

Figure 11 also includes the results of a parallel series of experiments in which nuclei were first exposed to potassium ions and then distributed as before, i.e., one half to a K^+ medium and the other half to an Na^+ medium. As expected, nuclei that did not receive sodium at any time failed to accumulate the amino acid (curve $K^+ \rightarrow K^+$). The corresponding curve in sodium ($K^+ \rightarrow Na^+$)

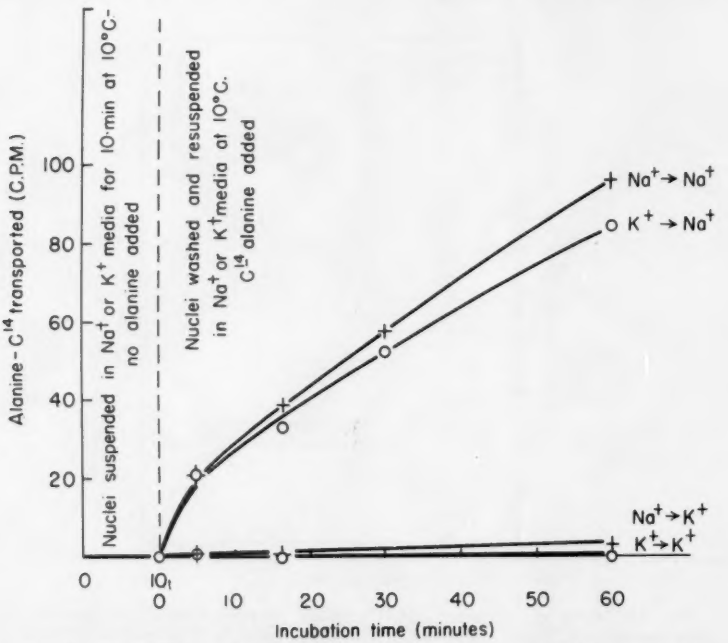

FIG. 11.—Failure of nuclei to retain the sodium ions necessary for amino acid incorporation. Nuclei were preincubated for 10 minutes at 10° in the sodium-rich medium described in Table 2, or in the equivalent all-potassium system. No C^{14}-alanine was present during this preincubation period. The nuclei were then washed by centrifugation. Half of the nuclear sediment was resuspended in the usual Na^+ medium; the other half was added to a K^+ medium. The time course of subsequent alanine-1-C^{14} transport into the nuclear "pool" is shown for these different conditions. Note the failure of nuclei previously exposed to Na^+ to accumulate alanine later in an all-K^+ system (curve $Na^+ \rightarrow K^+$).

is of special interest because it shows that the effect of sodium ions in promoting alanine transport is virtually instantaneous.

Tests for Sodium Ion Egress during Amino Acid Transport.—Some preliminary attempts have been made to test whether sodium ions are lost from the nucleus at the time alanine enters the nuclear "pool." In these experiments isolated nuclei were incubated with $Na^{22}Cl$, centrifuged, and then resuspended in the usual incubation medium. Some of the nuclear suspensions received a supplement of L-alanine; others served as "controls." After incubation for 15 or 30 minutes at 10°, both nuclei and media were assayed for their content of radioactive sodium. It was found that the addition of the amino acid did not significantly affect either the residual Na^{22} content of the nuclei, or the rate of loss of Na^{22} into the medium. The result suggests that amino acid transport does not involve an exchange of sodium ions for incoming alanine, but it should be stressed that the

relatively small extent of amino acid transport and the poor retention of sodium ions by isolated nuclei make it difficult to study exchange in this way, and further work on the problem is necessary.

Tests for Energy-Dependence of the Amino Acid Transport Reaction.—It has already been pointed out that the peak C^{14}-alanine concentration of the nuclear "pool" probably exceeds that of the surrounding medium. Since the transport and accumulation of an amino acid against a concentration gradient would be expected to require the expenditure of energy, several attempts were made to demonstrate an ATP-dependence for nuclear amino acid transport.

(a) *Effect of extracting nuclear ATP:* The first of these tests was based on an earlier observation that the extraction of thymus nuclei with dilute acetate buffers at pH values below 5 removes the nuclear mononucleotides, including ATP.[11] This effect is relatively specific for acetate, because a similar extraction with succinate or citrate buffers at the same pH values does not remove nuclear ATP. Using this extraction procedure, it became possible to test for alanine-1-C^{14} transport in nuclei which had lost all of their mononucleotides, and it was found that nuclei which were briefly treated with acetate at pH 4.5 had completely lost the ability to accumulate radioactive alanine. On the other hand, nuclei treated with succinate at the same pH value had normal rates of alanine-1-C^{14} transport. The results suggested a correlation between the level of ATP in the nucleus and its capacity for amino acid transport. (This evidence, however, must be considered as no more than tentative, because other experiments have shown that acetate extraction adversely affects the state of the nuclear "pool;" for example, nearly half of the C^{14}-alanine can be removed from a prelabeled nuclear "pool" by washing with acetate buffer under these conditions.)

(b) *Effect of inhibiting nuclear ATP synthesis:* In further attempts to relate nuclear ATP reserves to the process of amino acid transport, we made use of several inhibitors of nuclear phosphorylation.[11, 40] Histones, for example, are potent inhibitors of respiration and phosphorylation in isolated thymus nuclei.[41] It was found that the arginine-rich histone of calf thymus (Fraction I of Daly and Mirsky[42]) when added to nuclear suspensions, inhibits both ATP synthesis and amino acid transport. At a concentration of 1.5 mg/ml histone I reduced alanine entry into the nuclear "pool" by 40 per cent. This effect of histone is not due to dissolution of the "pool," because C^{14}-alanine previously taken up into nuclei is not removed by histone treatment. (The finding that added histone does not lead to extensive leakage of low molecular weight components from the isolated nucleus is in contrast to recent findings with bacterial cells[43] in which an extensive loss of nucleotides occurs when histones are added to the medium.)

Nuclear phosphorylation can be inhibited in other ways; dinitrophenol, for example, has been shown to block nuclear ATP synthesis *in vitro*.[11] If thymus nuclei are isolated in the presence of 0.002 *M* dinitrophenol, their respiration and phosphorylation are both suppressed and alanine transport is subsequently reduced to less than 50 per cent of that observed in "control" nuclei. Similar effects are observed when potassium cyanide is added to suppress nuclear ATP synthesis.

Finally, the effect of deoxyribonuclease was tested, because it is known that ATP synthesis in thymus nuclei is DNAase-sensitive.[40] It was found that when 40 per cent of the nuclear DNA was removed by enzyme treatment, the initial rate of

alanine transport fell by 37 per cent; i.e., from 0.281 μgm per 15 minutes in "control" nuclei to 0.176 μg/15 minutes in DNAase-treated nuclei. Moreover, when ATP synthesis was restored to DNAase-treated nuclei by the addition of polyanions[14] the rate of alanine transport increased to 0.249 μg/15 minutes, or 89 per cent of the rate observed in the "controls."

Thus the results of the inhibitor studies support the view that the transport of amino acids into the nucleus is ATP-dependent. Since it is not known whether ATP is consumed in this process, it remains to be seen whether this energy-dependence is direct or indirect.

Effects of Pyridoxal, Pyridoxal Phosphate, and Insulin on Alanine Transport.—Riggs *et al.*[5] have shown that the active transport of amino acids into cells is stimulated by the addition of pyridoxal. For this reason we tested the effect of the vitamin on nuclear amino acid transport. Both pyridoxal and pyridoxal phosphate were tested at a final concentration of 1 μmole per ml. Measurements of C^{14}-alanine transport into the nuclear "pool" showed no significant increase in radioactivity due to the addition of either pyridoxal or its phosphorylated derivative (Table 4).

TABLE 4

EFFECT OF INSULIN, PYRIDOXAL CHLORIDE, AND PYRIDOXAL PHOSPHATE ON ALANINE-1-C^{1} TRANSPORT IN NUCLEI

Expt.	Conditions*	C^{14}-alanine Content (μgm) of the "Pool" at† 15 minutes	30 minutes	60 minutes
1.	"Control" nuclei	0.197	0.353	0.474
	Nuclei + 0.5μg insulin	0.235	0.373	0.559
	Nuclei + 5.0 μg insulin	0.246	0.380	0.614
2.	"Control" nuclei	0.205	0.320	0.499
	Nuclei + pyridoxal chloride	0.204	0.322	0.491
	Nuclei + pyridoxal phosphate	0.197	0.336	0.475

* Incubation conditions as in Table 3. Beef insulin (8 units per mg) dissolved in 0.1 *M* phosphate = 0.25 *M* sucrose at 10 μgm/ml and 100 μgm/ml. Pyridoxal phosphate and pyridoxal chloride dissolved in 0.1 *M* phosphate -0.25 *M* sucrose at 4 μMolar. Final concentration of vitamin in incubation medium = 1 μMolar.
† "Pool" size expressed as μgm per 20 mg nuclei (dry weight).

Because the action of insulin is widely regarded as affecting the permeability of cells to various substrates, we examined the effect of this hormone on alanine transport into isolated nuclei. Insulin was added to the usual incubation medium to give a final concentration of 0.2 unit per ml. Measurements of alanine-1-C^{14} transport into the nuclear "pool" showed that insulin-treated nuclei were about 20 per cent more active than nuclei which had not received the hormone supplement. A similar effect of insulin in stimulating glycine transport into cut diaphragm muscle has recently been reported by Battaglia *et al.*[44]

SODIUM EFFECTS ON NUCLEIC ACID SYNTHESIS IN ISOLATED NUCLEI

Some degree of sodium ion dependence has also been demonstrated for nucleic acid synthesis in isolated thymus nuclei. It was shown by Breitman and Webster that the incorporation of glycine-2-C^{14} into nucleic acid purines is four to five times higher in nuclear suspensions containing sodium ions than in suspensions containing the equivalent potassium ion concentration.[3] This effect can now be seen to be due to the specific stimulation by sodium of glycine transport into the nuclear "pool." The experiments now to be described are concerned with the incorporation of purines, pyrimidines and their nucleosides into the DNA and RNA of

isolated thymus nuclei, and with the role of sodium ions in this aspect of nuclear metabolism.

Sodium Effects on Thymidine-2-C^{14} Incorporation into DNA.—The utilization of thymidine-2-C^{14} for DNA synthesis by isolated nuclei was first shown by Friedkin and Wood.[45] In studying this incorporation we compared the levels of C^{14}-uptake

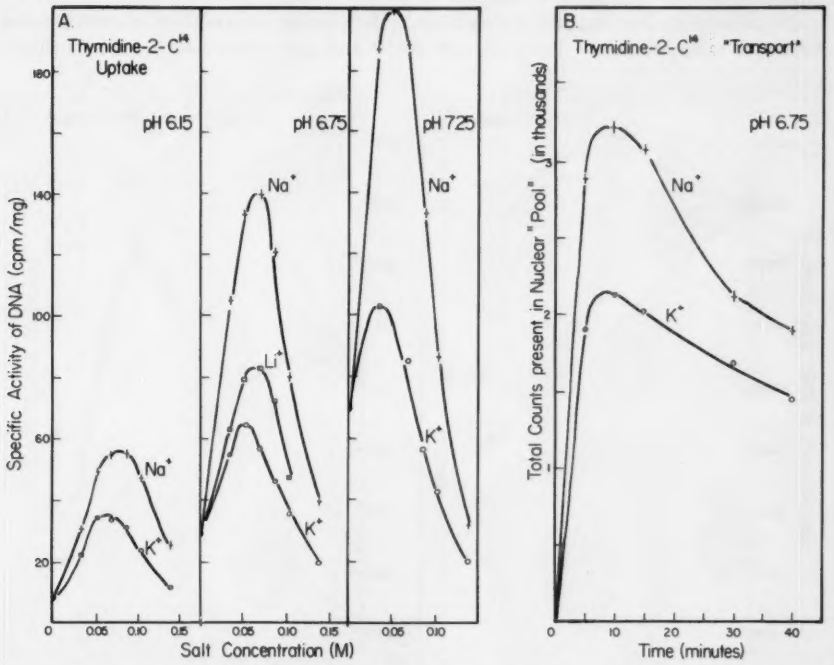

FIG. 12A.—Comparative effects of different monovalent cations on thymidine-2-C^{14} incorporation into the DNA of isolated thymus nuclei. The specific activity of the DNA (cpm per mg) after 60 minutes' incubation at 37° is plotted as a function of the salt concentration of the medium. The incubation mixture contained 1.0 ml of nuclear suspension, 0.5 ml of 0.1 *M* potassium phosphate buffer in 0.25 *M* sucrose (pH 6.15, 6.75, 7.25), 0.4 ml 0.1 *M* glucose containing 10 μmoles $MgCl_2$ and the indicated cations (as chlorides), and 0.1 ml H_2O containing 100 μg thymidine-2-C^{14} (1.1 mc/mmole).

FIG. 12B.—Comparison of thymidine-2-C^{14} "transport" in Na^+ and K^+ media. Incubation conditions as in Figure 12A. The specific activity of the nuclear "pool" is plotted as a function of the time of incubation at 37°.

in sodium and potassium containing media at different pH values. The results of this comparison are shown in Figure 12*A*, in which the specific activity of the DNA (after 60 minutes' incubation in the presence of thymidine-2-C^{14}) is plotted against the salt concentration of the medium. (The experimental details are given in the legend to the figure.) It is evident that sodium ions can more than double the rate of thymidine incorporation into DNA.

This increase is due, at least in part, to the specific stimulation by sodium ions of thymidine transport into the nucleus. The transport effect is shown in Figure 12*B*, in which the thymidine-2-C^{14} content of the nuclear "pool" is plotted against

the time of incubation at 37°. It is clear that the rate of thymidine entry into the nucleus is sodium dependent.

Figure 12*A* also includes the results obtained in the presence of equivalent concentrations of lithium. In agreement with the earlier findings on amino acid incorporation into nuclear proteins, lithium was found to be partially effective as a sodium-ion substitute.

Ion Effects on Nuclear RNA Synthesis.—(a) *Sodium stimulation of adenine and adenosine incorporation:* Both adenine-8-C^{14} and adenosine-8-C^{14} can be utilized

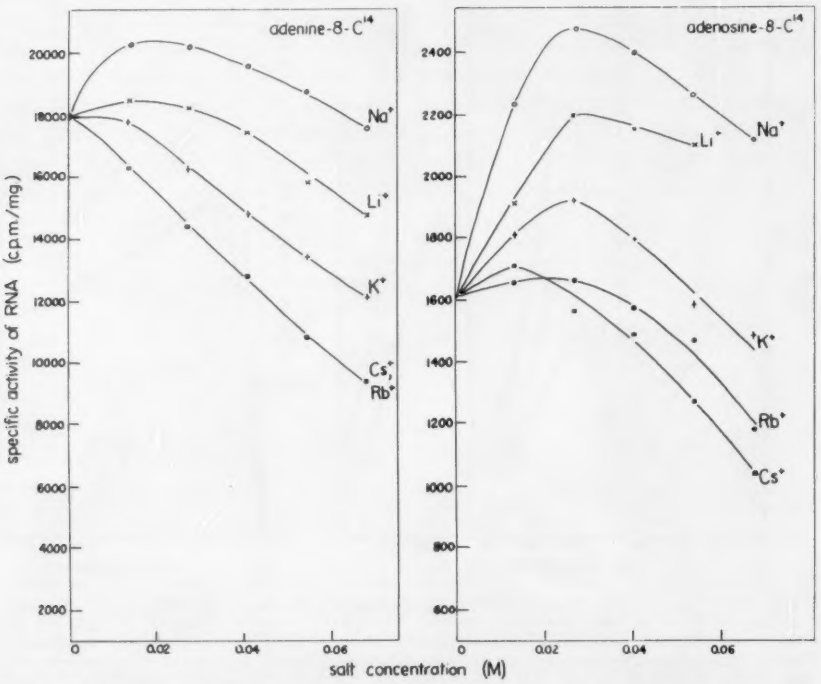

FIG. 13.—Comparative effects of different monovalent cations on the incorporation of adenine-8-C^{14} (A) and adenosine-8-C^{14} (B) into nuclear ribonucleic acid. In both cases the specific C^{14}-activity of the total nuclear RNA after 60 minutes' incubation at 37° is plotted against the salt concentration of the medium. Incubation conditions as in Figure 1, using 50 μg adenine-8-C^{14} (Sp. Act. 3.8 mc/mmole) or 480 μg adenosine-8-C^{14} (Sp. Act. 0.18 mc/mmole).

by isolated thymus nuclei for ribonucleic acid synthesis.[14–16] Very little incorporation into DNA occurs, since it has been shown that over 90 per cent of the total counts present in acid-washed nuclei after labeling experiments can be removed by ribonuclease digestion.[15]

The effects of different monovalent cations on the incorporation of radioactive adenine and adenosine into nuclear ribonucleic acid are illustrated in Figure 13, which plots the specific activity of the total RNA against the salt concentration of the medium. In both cases it can be seen that sodium ions must be present in the medium for optimal purine incorporation into RNA. As before, lithium was

found to be partially effective as a sodium substitute; but rubidium and caesium are definitely inhibitory to nuclear RNA synthesis.

As in the previous experiment with thymidine-2-C^{14}, the sodium effect on adenosine-8-C^{14} uptake is largely due to a stimulation of adenosine transport into the nuclear "pool." Thus, after 15 minutes at 37°, the C^{14}-activity of the "pool" was 159 cpm in sodium-treated nuclei, as compared with 131 cpm in potassium-treated nuclei. The increase in the rate of adenosine transport under these condi-

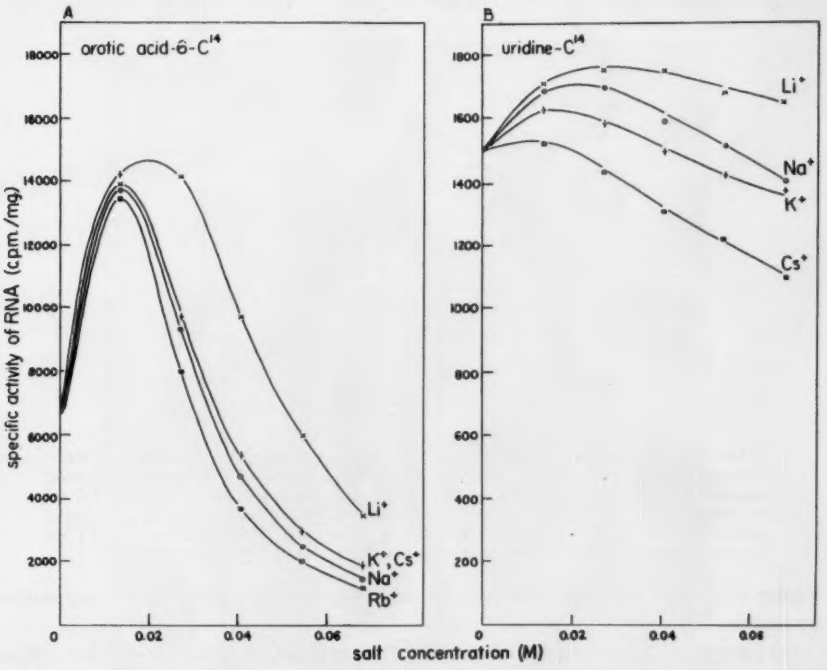

FIG. 14.—Comparative effects of different monovalent cations on the incorporation of orotic acid-6-C^{14} (A) and uniformly labeled C^{14}-uridine (B) into nuclear ribonucleic acid. In both cases the specific C^{14}-activity of the total nuclear RNA after 60 minutes' incubation at 37° is plotted against the salt concentration of the medium. The incubation mixture contained 1.0 ml of nuclear suspension, 0.5 ml 0.05 *M* potassium phosphate buffer in 0.125 *M* sucrose (pH 6.75), 0.4 ml 0.1 *M* glucose containing the indicated cations (as chlorides), and 0.1 ml H_2O containing 40 μg orotic acid-6-C^{14} (Sp. Act. 2.0 mc/mmole) or 27 μg uniformly labeled C^{14}-uridine (Sp. Act. 0.2 mc/mmole).

tions (21%) is sufficient to account for the observed increase in its incorporation into RNA.

(b) *Absence of the sodium requirement in orotic acid uptake:* Orotic acid-6-C^{14} is an effective precursor of the pyrimidines in nuclear RNA[2] and rapidly appears in both the uridylic and cytidylic acid components of the nucleic acid.[15] In studying the effect of monovalent cations upon this incorporation we found, to our surprise, that sodium ions were not necessary for optimal orotic acid uptake, and that potassium buffers provided an equally effective incubation medium. These results are summarized in Figure 14*A*. It is also of interest that lithium ions are now more

effective than sodium ions in promoting C^{14}-uptake, especially at the higher salt concentrations.

The absence of a specific sodium effect on orotic acid uptake into RNA pyrimidines can be correlated with the fact that sodium does not influence the rate of orotic acid transport into the nuclear "pool." A comparison of the time courses of orotic acid-6-C^{14} transport in sodium and potassium supplemented nuclei is shown in

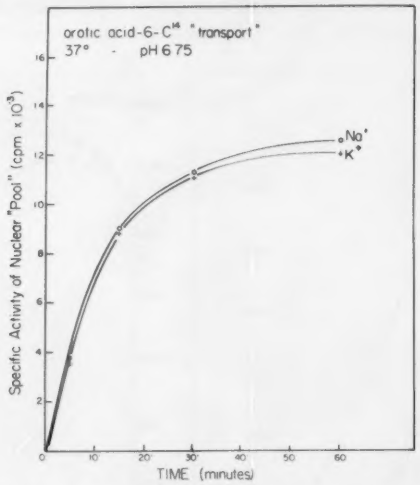

FIG. 15.—Comparative effects of sodium and potassium ions on orotic acid-6-C^{14} "transport" into the nuclear "pool." The specific activity of the "pool" is plotted against the time of incubation at 37°. The incubation mixture contained 1.0 ml of nuclear suspension, 0.5 ml 0.1 *M* potassium phosphate buffer in 0.25 *M* sucrose (pH 6.75), 0.4 ml 0.1-*M* glucose containing 10 μmoles $MgCl_2$ and 4 mg NaCl per ml, and 0.1 ml H_2O containing 40 μg orotic acid-6-C^{14} (Sp. Act. 2 mc/mmole).

Figure 15, and it is evident that the rate of entry into the "pool" is not augmented by sodium ions.

(c) *Ion effects on uridine incorporation:* The utilization of uridine-C^{14} for nuclear RNA synthesis has been shown to result in an incorporation of isotope into both the uridylic and cytidylic acids of the polynucleotide.[15] In this respect uridine uptake resembles the incorporation pattern of orotic acid. It differs from the latter, however, in its response to added cations. As shown in Figure 14*B*, uridine C^{14} incorporation into nuclear RNA proceeds better in sodium than in potassium-containing media. Also of interest is the stimulatory effect of lithium ions on uridine uptake, resembling that seen earlier for orotic acid incorporation. The lithium effect seems specific for the incorporation of pyrimidines, since neither adenine nor adenosine uptake increases in this way when sodium is replaced by equivalent amounts of lithium (Fig. 13).

Similar experiments on the effects of monovalent cations on uracil-C^{14} incorporation proved negative, because isolated thymocyte nuclei do not utilize the free pyrimidine for ribonucleic acid synthesis.

The experiments presented above point to a simple correlation. Where sodium ions stimulate the incorporation of a radioactive precursor into nucleic acid (as in

thymidine, adenine, and adenosine uptakes), one can also demonstrate a sodium dependent transport mechanism. In cases where sodium does not stimulate uptake (as in orotic acid incorporation into RNA), no sodium-dependent transport can be observed.

ON THE ROLE OF SODIUM IONS IN NUCLEAR METABOLISM

The preceding experiments have shown that protein and nucleic acid synthesis in isolated nuclei are sodium-dependent processes, and that this dependence reflects the operation of specific transport mechanisms. These mechanisms are

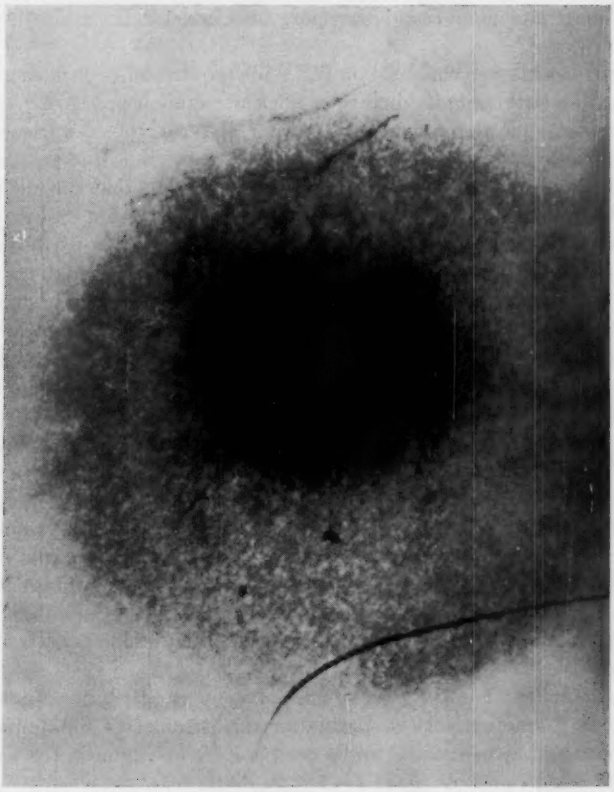

FIG. 16.—Autoradiograph showing the intracellular distribution of Na^{22+} after brief exposure of frog oocytes to media containing $Na^{22}Cl$. The high concentration of radioactive sodium in the nucleus is indicated by the high grain density over the nuclear area in this cell section.

apparently enzymatic and they require sodium ions in order to transfer (or admit) amino acids and nucleosides from the medium to the intranuclear "pool."

The sodium effect in controlling the rates of protein and nucleic acid synthesis *in vitro* suggests that sodium plays an important role in the regulation of nuclear metabolism *in vivo*, and this conclusion finds extensive support in studies of the intracellular distribution of sodium ions. Several different cell types have been

shown to have high intranuclear sodium concentrations. In frog oöcytes, for example, the accumulation of sodium ions in the nucleus has been shown by direct analysis of isolated nuclei[46] and by autoradiography of cells exposed for brief periods to media containing $Na^{24}Cl$ (see ref. 47) or $Na^{22}Cl$ (see Fig. 16).[46] It has also been reported that avian erythrocyte nuclei and calf thymus nuclei (isolated in nonaqueous media) have higher sodium concentrations than the corresponding cytoplasmic fractions.[48] Thus, *in vitro* and *in vivo*, there is good evidence that sodium ions play a special role in nuclear function.

Cytoplasmic systems, on the other hand, seem to be largely potassium-dependent. Amino acid uptake by liver cytoplasmic particles, for example, specifically requires potassium ions,[4] and amino acid transport into intact cells is also a potassium-dependent process.[5]

The differences in ionic composition and ionic requirements of nucleus and cytoplasm point to a striking individuality of nuclear metabolism. Apart from raising many interesting questions about the nature and control of nucleocytoplasmic interactions, these findings invite speculation on the origin of the unique biochemical properties of the cell nucleus. It is, of course, possible that the nucleus obtains its sodium directly from the cytoplasm. It may be suggested, however, that the nucleus is in frequent or continuous direct contact with the extracellular fluids (also a high sodium system). This contact could occur if infoldings of the cell membrane formed channels to the perinuclear space (as can be seen in some cells[49, 50]) or if vesicles, such as those formed during pinocytosis, progressed as far as the nuclear membrane. This type of contact would explain the rapid penetration of the diaminoacridine dyes into the nuclei of living cells.[51, 52]. In a more speculative vein, it may be suggested that nucleus and cytoplasm may represent a later evolutionary stage of what was originally a symbiotic relationship between primitive organelles, one representing a sodium system, and the other dependent on the potassium in its environment. Whatever the origin of the difference between nucleus and cytoplasm, the specific ionic requirements of the two systems offer new possibilities for the control and understanding of cell metabolism and function.

Summary.—The incorporation of amino acids into the proteins of isolated thymus nuclei is sodium-dependent. This dependence reflects the operation of sodium-specific "transport" or "gate" mechanisms which admit amino acids into the intranuclear "pool."

Amino acid transport into the cell nucleus has many attributes of an enzymatic (or "permease") reaction. It is specific for the L-isomer of the amino acids; it has a pH optimum at neutrality, and a temperature optimum in the physiological temperature range.

Competition studies indicate that specific receptor sites are concerned with the transport of different amino acids. These sites can readily discriminate between the D- and L-isomers of the appropriate amino acids, but close structural analogues such as L-isoleucine or glycine can competitively occupy the sites involved in valine or alanine transport, respectively.

The transport reaction appears to be energy dependent, since it is inhibited by agents which block nuclear ATP synthesis.

Similar sodium-dependent transport mechanisms have been observed for thymidine, adenosine, and adenine uptake. However, orotic acid incorporation into

nuclear RNA does not require sodium ions, nor is there any evidence for sodium-dependent orotic acid transport.

The nuclear requirement for sodium ions contrasts with the potassium dependence of cytoplasmic systems, and in living cells, the nucleus maintains a high Na^+ concentration. The origin of the nuclear sodium and its role in controlling protein and nucleic acid synthesis are discussed.

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*ON THE ACTION OF HORMONES WHICH ACCELERATE THE RATE OF OXYGEN CONSUMPTION AND FATTY ACID RELEASE IN RAT ADIPOSE TISSUE IN VITRO**

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It has become apparent from recent studies in this laboratory and those of others that certain hormones have the common property of stimulating three different metabolic processes in rat adipose tissue *in vitro*. Adrenaline, ACTH, glucagon, and TSH all accelerate in this tissue the rate of oxygen consumption, glycogen breakdown, and fatty acid release (Table 1). We wish to present here data which lend additional support to these observations and which permit the addition to this list of the fat mobilizing substance (F. M. S.) reported by Chalmers *et al.*[14] Data are also presented on the ability of the two peptides prepared from pituitary by Astwood *et al.*[30] to stimulate oxygen uptake *in vitro* by adipose tissue from the rabbit but not from the rat.

TABLE 1

A List of Hormones which Accelerate Oxygen Uptake, Fatty Acid Release, and Glycogen Breakdown in Adipose Tissue with Pertinent References

Hormone	Oxygen uptake	Fatty acid release	Glycogen breakdown
ACTH	2, 3, 4	13, 18, 19, 20	10, 11, 21
Adrenaline	1, 2, 3	1, 9, 15, 16	10, 11, 21
Glucagon	2, 5	5, 12	5, 10, 11, 21
F.M.S.	7	7, 14	10
TSH	6, 7	4, 6	10
Growth hormone preparation*	2, 8	17, 18	10

* Recent work[4] indicates that the action of the growth hormone preparation is most likely due to its content of TSH.

As described by Chalmers *et al.*,[14] F.M.S. is excreted in the urine of humans who are fasting and actively mobilizing and utilizing fat. It is polypeptide-like in nature, causes transient hypoglycemia, ketonaemia, and increased mobilization and catabolism of fat upon injection into mice. It is an effective stimulator of the release of free fatty acids from rat adipose tissue *in vitro*. Though the pituitary appears to be necessary for its production, F.M.S. is apparently neither growth hormone nor corticotropin. The pituitary peptides of Astwood *et al.*[30, 31] stimulate

the release of fatty acids from the adipose tissue of rabbits but are much less effective in this regard on the adipose tissue of rats.

An explanation for the dual stimulatory action of all these hormones on oxygen consumption and fatty acid release in adipose tissue is offered. It is proposed that the stimulation of oxygen uptake is secondary to the enhanced liberation of fatty acids by these hormones and that it is a reflection of an increased consumption of high energy phosphate in a process whereby re-esterification of the fatty acids occurs.

Methods.—Male albino rats purchased from the Holtzman Company and weighing 140–180 gm were employed. The precautions observed in the care and handling of animals as well as the procedure for removal of the epididymal fat body have been described previously.[22, 24] Oxygen consumption was determined manometrically at 37° with air as the gas phase. The basic incubation medium was 3.0 ml of Krebs-Ringer phosphate medium, pH 7.4[23] modified to contain one-half the recommended calcium. When glucose was present its concentration was 3 mg per ml. In some experiments human serum albumin (fraction VR, Protein Foundation) was dissolved in the medium to yield a 5% solution and the pH restored to 7.4 by the addition of 0.15 ml of 1 *N* NaOH per 10 ml. The center well of the flasks (15–17 ml total capacity) contained 0.2 ml of 20% KOH and a roll of filter paper. Net gas exchange was determined as described previously.[24] Free fatty acid release was measured by the method of Dole[25] with slight modification, glucose by the method of Somogyi-Nelson,[26, 27] and lactate by the method of Barker and Summerson.[28]

The sample of F.M.S. employed was kindly donated by T. M. Chalmers. The insulin was a crystalline preparation (Lot #466368) supplied through the courtesy of Eli Lilly and Co. E. B. Astwood generously furnished samples of pituitary fractions I, and I and II, as well as α-MSH. The sample of TSH assayed six units per mg and was the gift of A. E. Wilhelmi.

Results.—The effect of the addition of F.M.S. upon the oxygen consumption of adipose tissue is illustrated by the data presented in Figure 1. Here two different experiments are shown, in each, paired tissues from a single rat were employed. In one experiment, portrayed in the left half of Figure 1, one piece of tissue was placed into medium that contained 0.1 unit (4 μg) of insulin per ml and the other piece into medium without insulin. Glucose was present in both flasks. The rate of O_2 consumption per 100 mg wet weight of tissue during an initial 30-minute period was determined, and then F.M.S. was added from the side arm of the flask to yield a final concentration of 33 μg per ml. In both flasks a prompt stimulation of oxygen uptake occurred which was much more marked in the presence of insulin than in its absence. An inhibition of O_2 uptake soon set in when insulin was absent so that by the third hour of the experiment the rate had fallen to about 50 per cent of the initial value. Previous experiments from this laboratory have shown that the rate of O_2 consumption of untreated tissues remains nearly linear over a three-hour period and that under conditions similar to those used here the addition of insulin alone has little effect upon the O_2 consumption.[1, 8]

In the experiment shown in the right half of Figure 1, one piece of tissue was placed into medium containing glucose and 5 per cent albumin while the paired piece of tissue was incubated in medium containing glucose and no albumin. Again after an initial 30-minute period F.M.S. was added to both flasks to yield a final concentration of 33 μg per ml. In the presence of albumin the stimulation of O_2 uptake that results initially is maintained. In contrast, a progressive inhibition in rate is observed with the paired tissue. These results are similar to those seen when the same type of experiments are performed with adrenaline.[1]

In Table 2 are given the results of experiments in which the concentration of

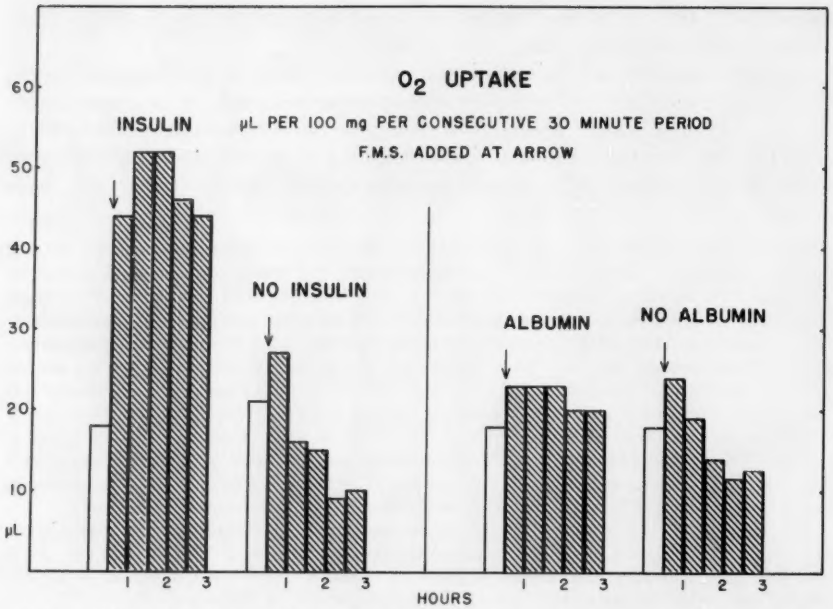

FIG. 1.—The effect of fat mobilizing substance (F.M.S.) on the oxygen consumption of rat adipose tissue. Experimental conditions as described in the text. The concentration of F.M.S. was 33 μg per ml, and when present, insulin 0.1 unit (4 μg) per ml, albumin 50 mg per ml.

F.M.S. was varied. Each experiment was performed in a manner similar to the ones shown in Figure 1, with paired tissues from a single rat being employed in each case. Unless otherwise stated, glucose (3 mg/ml) and insulin (0.1 u/ml) were present from the start. Under these conditions the increase in rate of O_2 consumption produced is a function of F.M.S. concentration within the range 1.0 to 33 μg/ml. Concentrations higher than 33 μg/ml were not tried since the supply of F.M.S. was limited. Previous experience with other hormones has shown that the limit of the tissue's ability to consume O_2 is reached in the vicinity of a 200 per cent in-

TABLE 2

THE EFFECT OF ALTERATIONS IN F.M.S. CONCENTRATION AND MEDIUM COMPOSITION ON THE OXYGEN CONSUMPTION OF RAT ADIPOSE TISSUE

Experiment	F.M.S., μg/ml	O_2 Uptake,* μl per hr per 100 mg Initial	After F.M.S.	% Change	Comment
1 A	33.3	35	105	+200	
B	3.3	34	67	+97	
2 A	3.3	46	90	+96	
B	3 3	36	42	+17	No glucose
3 A	1.0	26	30	+15	
B	1.0	28	31	+11	No insulin
4 A	33.3	34	104	+206	
B	33.3	40	20	−50	No insulin
5 A	10.0	30	18	−40	No insulin
B	10.0	33	44	+33	5% albumin and no insulin

* The initial rate is determined from a 30-minute period before the addition of F.M.S. while the rate after F.M.S. represents the value for the third hour of the incubation.

crease in rate. The data in Table 2 also show that the presence of glucose is necessary for the effect of insulin to be seen.

In experiment 5 of Table 2, the free fatty acids of the medium and tissue at the end of the 3-hour incubation were determined. In 5*A* the 3.0 ml of medium contained a total of 0.39 μmol of fatty acid per 100 mg of tissue, while each 100 mg of the tissue itself contained 1.71 μmol. When albumin was present (5*B*) the medium gained a total of 2.44 μmol of fatty acid per 100 mg of tissue and the tissue itself contained 1.19 μmol per 100 mg.

TABLE 3

THE EFFECT OF F.M.S. ON THE METABOLISM OF RAT ADIPOSE TISSUE IN A BICARBONATE BUFFERED MEDIUM

	(Experiment 1)		(Experiment 2)	
	Control	F.M.S.	Control	F.M.S.
Net gas exchange	−1.2	−0.4	−2.0	−0.3
Glucose uptake	0.81	1.25	0.62	1.54
Lactate production	0.32	0.50	0.28	0.51
Free fatty acid changes	−0.36	1.93	−0.30	1.41

F.M.S. final concentration 10 μg/ml. All values in μmol per 100 mg of tissue wet weight per 3-hour incubation period. Each flask contained 1.5 ml total fluid volume. Paired tissue from a single rat was employed in each experiment. The medium was Krebs-Ringer bicarbonate modified to contain one-half the recommended calcium. Albumin (5%) and glucose (1.5 mg per ml) were present in all cases. The albumin employed contained a small amount of fatty acid which resulted in the medium's showing an initial value of 0.9 μmol of fatty acid per ml.

The effect of F.M.S. on the metabolism of adipose tissue in a bicarbonate buffered medium containing albumin is shown in Table 3. The amount of fatty acid released into this medium at this concentration of F.M.S. (10 μg/ml) is in reasonable agreement with that reported by Chalmers *et al.*[14] There is a slightly greater uptake of glucose and output of lactic acid by the tissue in the presence of F.M.S. The net gas exchange of the tissue is also less negative with F.M.S. present. This

TABLE 4

A COMPARISON OF THE EFFECTS OF CERTAIN ANTERIOR PITUITARY FRACTIONS ON THE OXYGEN CONSUMPTION OF RAT AND RABBIT ADIPOSE TISSUE

Substance	Concentration (μg/ml)	Per Cent Increase in Oxygen Consumption	
		Rat	Rabbit
Peptide I	5	0 (1)	98 (1)
Peptide I & II	5	0 (6)	87 (5)
TSH	5	163 (6)	10 (5)
	1	156 (12)	
	0.1	50 (4)	
α-MSH	5.0	39 (1)	134 (1)

Values are the average of the number of experiments indicated by the figures in parentheses. Experiments were run in the manner previously described with insulin (0.1 unit/ml) and glucose present (3 mg/ml). In some experiments albumin was also present (5%) but it was without effect upon the response obtained. Epididymal fat body was the source of tissue in the rat experiments while in the rabbit perirenal and omental adipose tissue were employed.

may be a reflection of CO_2 release from the bicarbonate of the medium due to the increased acid production. In other experiments in which the final concentration of F.M.S. was 33 μg per ml the net gas exchange of the tissue also remained negative. Moreover, no effect of F.M.S. at this concentration was found on the net gas exchange response of the tissue to 0.1 unit of insulin per ml. In this respect the action of F.M.S. is unlike that of adrenaline.[29]

Astwood *et al.*[30] have described the preparation from porcine anterior pituitary lobes of two peptides which are active in releasing fatty acids from the perirenal or omental adipose tissue of rabbits. They are relatively inactive in this respect on the epididymal fat pad of rats.[31] In Table 4 data are presented which show that

these fractions are capable of stimulating oxygen consumption of adipose tissue from the rabbit but not from the rat. Also presented in this table are data on the action of TSH. This substance is very active in stimulating the oxygen consumption of rat adipose tissue but is relatively inactive on rabbit adipose tissue. A species difference thus appears to exist in the response of adipose tissue to these pituitary fractions. A similar situation may exist with regard to α-MSH but the limited amount of this material available to us permitted only one experiment to be performed.

Discussion.—It is possible to offer an explanation for the effect of the hormones listed in Table 1 upon the oxygen uptake of adipose tissue which is based upon their ability to increase the production of unesterified fatty acids in this tissue. The schema in Figure 2 is presented to aid in this explanation. The accumulated fat

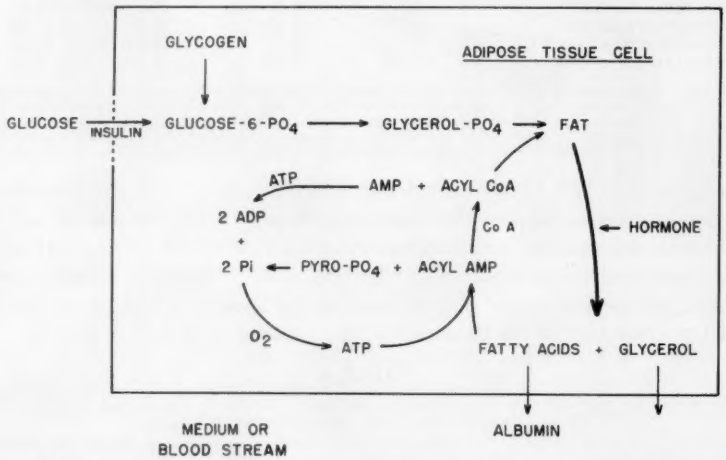

FIG. 2.—A schema offered as an aid in the explanation of the concomitant stimulation of oxygen consumption and fatty acid release in adipose tissue by certain hormones.

within the adipose tissue cell is visualized as being in a dynamic steady state with respect to the pool of fatty acids in the tissue. Thus under normal conditions a continual hydrolysis of fat to fatty acids and glycerol is envisioned which is balanced by a re-esterification of the fatty acids to form new triglycerides. In the presence of the hormones under discussion an acceleration of the lipolysis of fat to fatty acids and glycerol takes place. As fatty acids begin to accumulate in the tissue, two pathways are portrayed as open to them; they may leave the tissue to enter an albumin-fortified medium or the blood stream, or they may undergo re-esterification at an increased rate. In addition, though not indicated in Figure 2, they may also proceed through acyl CoA to steps leading to their oxidation. The proportion of fatty acid following each of these pathways will depend on certain other factors. Now as shown in Figure 2 the re-esterification process requires energy which is furnished by ATP. The resulting breakdown of ATP to ADP and inorganic phosphate (Pi), as indicated, can in turn trigger an increase in oxygen consumption for the regeneration of ATP. The fuel for this oxidative process may be fatty acid, glucose, or both.

The maximum stimulation of oxygen consumption by any of these hormones occurs when both insulin and glucose are present in the incubation medium. The primary reason for this effect is believed to be due to the stimulation by insulin of glucose uptake by the cell and the consequent increased conversion of glucose to glycerol phosphate. Without an adequate supply of this compound re-esterification of fatty acids presumably can not proceed.[32] The glycerol produced during lipolysis apparently cannot be re-utilized to furnish glycerol phosphate due to the absence of glycerol kinase in this tissue.[33] Shapiro *et al.*,[34] Cahill *et al.*,[35] and Lynn *et al.*[3] have shown that radioactive glycerol is not readily converted to either triglyceride glycerol or CO_2 by adipose tissue incubated *in vitro*. It has also been shown that adrenaline[3, 15] and glucagon[5] increase the release of glycerol in addition to free fatty acids from adipose tissue incubated *in vitro*. Hagen[36] has also shown that injection of noradrenaline into rabbits produces a parallel rise in blood free fatty acids and glycerol.

Although all the hormones which cause a release of fatty acids also stimulate oxygen uptake, the patterns of their action are not identical. F.M.S., ACTH, and adrenaline are similar in their action. The addition of any of these compounds may produce a biphasic response in which a stimulation of oxygen uptake occurs first followed by an inhibition. The inhibition sets in more promptly and is more severe when high concentrations of the hormones are employed or when glucose is absent from the medium. The inhibition can be delayed or even prevented by the simultaneous addition of insulin in the presence of glucose. The inhibition may also be diminished or prevented by the inclusion of albumin in the incubation medium. These facts strongly suggest that the inhibition of oxygen uptake is the result of an excessive accumulation of fatty acids within the tissue due either to their failure to leave the tissue or to the inability of the re-esterification process to keep pace with the rate of fatty acid production. Direct analyses of the tissue have shown this to be true.[1, 37] The cause of this inhibition may be related to the fact that fatty acids are inhibitors[38, 39] of the electron transmitter system of the mitochondria. It is also possible that at least part of the initial stimulation of O_2 consumption is due to the ability of fatty acids to uncouple oxidative phosphorylation.[40] The diminution in the supply of ATP that would thus result, would however in time lead to a failure of the re-esterification process with a consequent greater accumulation of fatty acids.

On the other hand with TSH or glucagon an inhibition never occurs and one only sees a stimulation of oxygen consumption. It is believed that this variation in pattern of action of these different hormones *in vitro* is a reflection of their respective potencies as releasers of fatty acids on the one hand and their ability on the other hand to bring about an uptake of glucose by adipose tissue. If the rate of production and accumulation of fatty acids within the tissue exceeds the rate of formation of either glycerol phosphate or of high energy phosphate needed for the re-esterification process then inhibition of O_2 uptake will result.

There is ample evidence in the recent literature to indicate that ACTH and adrenaline release fatty acids either *in vivo* or from adipose tissue incubated *in vitro*. This aspect has been reviewed very adequately (cf. 41 and 42). Also well documented is the fact that the release of fatty acids by these hormones is diminished if both insulin and an ample glucose supply are available.[16, 42] This dim-

inution in release of fatty acids appears not to be due to an inhibition of the conversion of fat to fatty acid but to an acceleration of the process whereby these acids are re-esterified to triglycerides. For example several workers[34, 43, 44] have reported that adipose tissue incubated *in vitro* incorporates labeled free fatty acids from the medium into neutral lipid. Bally *et al.*[43] have shown that the rate of esterification of fatty acids is enhanced by the presence of glucose in the medium and that if insulin is added in addition, a further stimulation in rate may be observed. These workers have suggested that this effect of glucose reflects its need as a source of glycerol phosphate for the esterification process. Evidence that the triglycerides of adipose tissue continuously undergo lipolysis to free fatty acids and the fatty acids are in turn re-esterified has been presented by Cahill *et al.*[45] These workers showed that when adipose tissue is incubated *in vitro* in the presence of radioactively labeled glucose, the glyceride glycerol rapidly became labeled. The amount of this label was out of all proportion to that which would be expected based solely on the *de novo* fatty acid synthesis measured simultaneously. In a later study Cahill *et al.*[46] showed that this incorporation of glucose carbon into glyceride glycerol was enhanced when adrenaline was added and an even further stimulation resulted if insulin was also present. A stimulation of glucose-incorporation into glyceride glycerol was also observed with ACTH and a growth hormone preparation.[18]

Of especial interest are the *in vivo* studies of Davidson *et al.*[47] These workers have reported that the injection of 1 mg of glucagon or 0.1 mg of epinephrine into a rat weighing 200–250 gm produces an increase of about 50 per cent in the rate of O_2 consumption one hour after the injection. This response is markedly reduced in adrenalectomized rats but can be restored by cortisone therapy. This latter point is of particular interest in view of the fact that free fatty acid release from adipose tissue is not induced by epinephrine in adrenalectomized rats[48] or dogs,[49] but can be restored in both cases by cortisone therapy. These findings suggest that the stimulation of oxygen consumption by adipose tissue *in vitro* by these agents may also occur *in vivo* and that a mechanism similar to that discussed here may be functioning. It should be pointed out however that in the intact animal an increase in oxygen consumption may not be solely due to adipose tissue. Fatty acids released into the blood stream by the action of these hormones may undergo re-esterification in other tissues (e.g., liver) with a concomitant increase in O_2 consumption by these tissues.

A process whereby fat is broken down to fatty acid and glycerol with a subsequent re-esterification of the fatty acid by way of glycerol phosphate furnished by glucose seems at first glance to be wasteful and useless. The question may be raised, however, as to whether this process might be utilized by homeothermic animals to generate heat for the maintenance of body temperature. The possible role of brown adipose tissue in this regard would be of especial interest since it is so abundant in hibernating animals and extremely rich in cytochromes.[50] George Cahill has made the interesting suggestion[51] that perhaps subcutaneous fat should be thought of not just as an insulating blanket but as an electric blanket.

It may be calculated that for each molecule of triglyceride broken down to glycerol and fatty acids seven high-energy phosphate bonds would be needed to reconvert the fatty acids back to triglyceride, two for each of the three fatty acids and one to provide the glycerol phosphate. If three high energy phosphate bonds

are formed for each atom of oxygen consumed, then in this process a shade over one molecule of O_2 is needed per molecule of triglyceride synthesized. With all these hormones, maximum increases in O_2 consumption of the order of 2–3 μmol per 100 mg of tissue per hour are observed on the average under optimum conditions. Hence, to account for this amount of O_2 comsumption the turnover of triglyceride would have to also be in the range of 2–3 μmol per hour per 100 mg of tissue or 6–9 μmol of fatty acid would be re-esterified.

Adipose tissue from young animals when stimulated by adrenaline *in vitro* has been reported to release 5 μmol of fatty acid per 100 mg per hour.[52] A smaller release of fatty acid is observed when tissue from older animals is employed. It is worthy of note that the oxygen consumption of adipose tissue from young rats also exceeds that from older animals.[1, 8] Whether these facts are the reflection of a higher rate of fatty acid re-esterification and perhaps of the higher basal metabolic rate of young animals can not be answered at this time.

As indicated in Table 1, all the hormones which stimulate the release of fatty acids from adipose tissue and increase its oxygen consumption also enhance the breakdown of glycogen in this tissue. The glycogenolytic action of adrenaline, glucagon, and ACTH in other tissues has been traced to their ability to produce adenosine-3′,5′-phosphate.[53] The question has therefore been raised[53] as to whether the formation of this compound might be responsible for the stimulation by these hormones of both glycogen and fat breakdown in adipose tissue. On the other hand, the question might be asked: does the formation of fatty acids and their re-esterification lead to the production of adenosine-3′,5′-phosphate? Is it possible that the acyl-AMP compound formed during the conversion of fatty acids to fat acts as a potential source for the formation of the cyclic derivative of AMP? If such were the case, then the need for glycerol phosphate in the re-esterification process would be automatically ensured by stimulation of glycogen breakdown. It should be noted that serotonin stimulates the breakdown of glycogen in adipose tissue, but does not cause release of fatty acids.[21] This finding has been confirmed and in addition serotonin has been found to be without effect upon the O_2 comsumption of adipose tissue.[10] Hence increased glycogen breakdown per se does not necessarily stimulate O_2 uptake.

Summary.—Previous observations which show that hormones capable of accelerating the release of fatty acids from adipose tissue also stimulate the oxygen consumption of this tissue *in vitro* are extended. The list of hormones which possess this dual action now includes adrenalin, adrenocorticotropin, glucagon, thyrotropin, certain growth hormone preparations, the urinary fat mobilizing substance of Chalmers *et al.*, and the two pituitary peptides separated by Astwood and coworkers. Some of these compounds show a species specificity in their dual action. It is suggested that the stimulation of oxygen consumption is secondary to the liberation of fatty acids by these hormones and is a reflection of an increased consumption of high-energy phosphate for the re-esterification of these fatty acids. The results are discussed in relation to observations in the literature which support this premise and to the possible significance of this seemingly wasteful process in the generation of heat for the maintenance of body temperature.

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Abbreviations used are: ACTH (adrenocorticotropin), AMP (adenosine monophosphate), ADP (adenosine diphosphate), ATP (adenosine triphosphate), CoA (coenzyme A), F.M.S. (fat mobilizing substance), TSH (thyrotropic hormone), Pi (inorganic phosphate), and α-MSH (melanocyte stimulating hormone).

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*EXPERIMENTS ON HEMOGLOBIN BIOSYNTHESIS**

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We have been concentrating lately on the chemical characterization of the mechanism of peptide linking, the terminal reaction in the polymerization of amino acids. For this purpose, the laying down of peptide chains on ribosomes was studied with aminoacyl soluble ribonucleic acids (sRNA's), the "active" amino acids, as the donors. Unfortunately, in the much used systems from liver[1] and *E. coli*[2] the protein formed is rather ill-defined. In this predicament, we turned our attention to the hemoglobin-producing system in reticulocytes.[3] As is well known, these cells may be obtained from rabbits appropriately injected with phenylhydrazine. In the resulting phenylhydrazine anaemia, hemoglobin-producing, but already enucleated, red cell precursors, the reticulocytes, replace up to 90 per cent of the normal synthetically inert red cells. A hemoglobin-producing cell-free system has been prepared from reticulocytes;[4] it resembles those from liver and *E. coli*, with the important difference that it produces a well-defined protein.

After it had become clear that the aminoacyl-sRNA represents the activated amino acid from which the peptide link is formed, a rather amazing nonspecificity of aminoacyl-sRNA from various sources[5] aroused our interest. Apparently any amino acid-carrying sRNA would indiscriminately react with mammalian or microbial ribosomes; for example, it was reported[1] that ribosomes from rat liver or mouse neoplasm would utilize *E. coli*-derived aminoacyl-sRNA for synthesis of protein as easily as their own sRNA.

To confirm the general compatibility of any aminoacyl-sRNA, it was decided to attempt hemoglobin synthesis by cross-reacting *E. coli* aminoacyl-sRNA with rabbit reticulocyte ribosomes. Such experiments have been successful and it will largely be the purpose of this paper to report on them. Furthermore, a preliminary analysis is presented of the components of the aminoacyl-sRNA-ribosome system from reticulocytes.

Methods.—Preparation of E. coli aminoacyl-sRNA: Soluble-RNA from *E. coli* was prepared by the phenol procedure developed by Acs.[6] The bacterial paste was suspended in an equal volume of 0.001 *M* Tris-HCl buffer, pH 7.2, containing 0.01 *M* magnesium acetate. The heavy suspension was shaken with an equal volume of redistilled phenol at room temperature for at least 1 hour. The mixture was centrifuged at 10,000 × *g* for 30 minutes, the water layer was removed, and the phenol layer was washed once with 5 ml of 0.001 *M* Tris buffer, pH 7.2. To the combined water layers, one-tenth the volume of 20 per cent potassium acetate solution was added, and the

RNA was precipitated with 2 volumes of ethanol at −20° and washed with 67 per cent ethanol. The precipitate mostly dissolved in 1 *M* NaCl solution, and a small residue was centrifuged off at 15,000 × *g* for 30 minutes. The supernatant was dialyzed overnight against distilled water and lyophilized.

To remove any attached amino acids, the sRNA preparation was incubated in 0.5 *M* Tris HCl, pH 8.8, for 45 minutes at 35°. For charging with amino acids, a typical reaction mixture contained in a final volume of 10 ml: an equimolar mixture of amino acids, 1 μmole of each amino acid, including uniformly labeled C^{14}-L-leucine (66×10^6 cpm/μ*M*); 0.4 ml of dialyzed 105,000 × *g* supernatant from alumina ground *E. coli;* 100 mg of *E. coli* sRNA; 1,000 μ*M* Tris HCl buffer, pH 7.2; 50 μ*M* magnesium acetate; 30 μ*M* ATP; 200 μ*M* PEP; and 0.4 mg of pyruvate kinase. After incubation for 10 minutes at 37°, the reaction was stopped by the addition of 10 ml of redistilled phenol and the charged sRNA was isolated as described. Aminoacyl-sRNA in the amount of 85 mg was obtained, which had a specific activity of 55,000 cpm/mg sRNA, or 0.83 mμ*M* leucine/mg of sRNA.

Preparation of ribosomes from rabbit reticulocytes: Essentially the method of Schweet *et al.*[4] was used. After lysing the washed cells with 4 vol of 0.005 *M* magnesium acetate for 5 minutes, 1 vol of 1.5 *M* sucrose-0.15 *M* potassium chloride was added and the 10,000 × *g* precipitate discarded. The supernatant was centrifuged for 1 hour at 105,000 × *g* and the ribosome pellet washed once with 0.35 *M* sucrose, 0.035 *M* $KHCO_3$-5% CO_2, 0.004 *M* $MgCl_2$, and 0.025 *M* KCl, pH 7.4 (Medium A).

Preparation of deoxycholate-washed (DOC) particles for transfer factor studies: Ribosomes, washed once in Medium A, were homogenized in a 0.5 per cent solution of DOC, centrifuged at 15,000 × *g* for 10 minutes, and the precipitate rejected. The ribosomes were collected by centrifuging at 105,000 × *g* for 3 hours. The supernatant was carefully decanted; the pellets were flushed five times with Medium A and redissolved by homogenizing lightly. Aggregated material was removed by a 10-minute spin at 15,000 × *g*. The final solution of ribosomes was made up to approximately 20 mg/ml in Medium A; it could be frozen, and stored well at −20°. The concentration of RNA was estimated by optical density determination at 260 mμ, assuming 1 mg/ml to be equivalent to an optical density of 24. Protein content was determined by the Lowry method[7] with bovine serum albumin as standard. Hemoglobin was estimated spectrophotometrically as cyanmethemoglobin.[8]

Assay for radioactivity: The protein precipitated in 5 per cent TCA was treated at 90° for 20 minutes with 5 per cent TCA, washed twice with 5 per cent TCA, once with ethanol/ether 3:1, and finally with ether. The dry material was dissolved in concentrated formic acid, transferred to planchets, weighed after evaporation of the formic acid, and counted. Appropriate corrections for self-absorption were made.

Materials.—ATP and GTP were products of Pabst Laboratories, Milwaukee. Phosphoenolpyruvate (PEP) silver barium salt and pyruvate kinase were obtained from C. F. Boehringer & Soehne, Mannheim, Germany. Uniformly labeled C^{14}-leucine with a specific activity of 88.5 μC/μ*M* was obtained from Volk Radiochemical Company, Chicago. Carboxymethyl (CM) cellulose was a product of Serva Entwicklungslabor, Heidelberg, Germany, and had a capacity of 0.72 mEq/gm. Trypsin was 2 × crystallized, salt-free protein, and was obtained from Worthington Biochemicals, Freehold, New Jersey.

CHARACTERISTICS OF THE AMINOACYL-SRNA-RIBOSOME SYSTEM

It has been found with liver ribosomes[5, 9] that the absolute amount of transfer depends largely on the concentration of ribosome; if the proportion between the amino acid-carrying sRNA and the ribosome is optimal, a very efficient transfer occurs. Occasionally it has been observed in liver preparations of this kind that up to 80 per cent of the added amino acid charged to the RNA may be transferred. The efficiency of transfer in the reticulocyte ribosome system is rather similar. As shown in Table 1, up to 66 per cent of the added leucyl-sRNA appears in the protein after 1 hour's incubation if the amount of added aminoacyl-sRNA is well tuned to the amount of particles. A fourfold increase in the amount of aminoacyl-

sRNA results in only a 1.4-fold increase in over-all incorporation.

In contrast to the liver system, under favorable conditions the reticulocyte particle releases most of the newly formed protein. The data in the last two columns of Table 1 indicate that the relative amount of hemoglobin shed into the supernatant depends on the absolute amount of aminoacyl-sRNA added. At a low level, percentage transfer may be high and relatively little is released, while at the highest level used, 80 per cent of the newly formed protein is liberated into the surrounding medium. The release of hemoglobin is obviously favored if the ratio of aminoacyl-RNA to ribosomes is high. This lends support to the conclusion by Bishop *et al.*,[10] and the even more convincingly presented conclusion by Dintzis[11] that globin chains form on the particle by a terminal addition of amino acids. It would then be expected that the number of chains that are completed and can be released will depend on the amount of aminoacyl-sRNA available. An increasing release with increasing availability of active amino acids therefore had to be expected and indeed was found. Morris and Schweet[12] have shown recently that the release of radioactive protein from previously labeled reticulocyte

TABLE 1

TRANSFER OF INCREASING AMOUNTS OF AMINO ACIDS FROM *E. coli* sRNA TO HEMOGLOBIN

No.	C^{14}-leucine-bound sRNA added		Over-all incorporation		Ribosomes		Supernatant	
	cpm	mμmole	cpm	%	cpm	%	cpm	%
1	880	0.1	530	61	372	70	158	30
2	26,200	3.0	17,300	66	5,250	30	12,050	70
3	105,000	12.0	30,350	29	6,150	20	24,200	80

Incubation conditions were the same as those in Table 3, except that 5 mg ribosomes, once washed in Medium A were used which did not require supernatant factor for activity. Increasing amounts of *E. coli* sRNA containing all amino acids including C^{14}-leucine were added; the specific activity was 8,800 cpm/mμ*M* leucine per mg of sRNA. The incubation was for 1 hour at 35°.

ribosomes is dependent on the presence of soluble enzymes and ATP in the complete system necessary for the incorporation of free amino acids. Neither ATP nor soluble enzymes, other than those present on the washed ribosomes, are required to release soluble hemoglobin in our experiment starting from *E. coli* aminoacyl-sRNA.

IDENTIFICATION OF HEMOGLOBIN DERIVED FROM *E. coli* AMINOACYL-SRNA

To test the synthesis of hemoglobin from *E. coli* sRNA-bound amino acids, we chose conditions where the larger amount of hemoglobin is separated from the particles. Obviously, for purification and further analysis, the free hemoglobin in solution is much more suitable than that which may be bound to particles. The following large-scale experiment was carried out: 50 mg of reticulocyte ribosomes washed once with Medium A, and 59 mg of *E. coli* sRNA charged, as described under *Methods*, with 18 amino acids and 49 mμ*M* C^{14}-L-leucine having a radioactivity of 3.24×10^6 cpm, were incubated for 60 minutes at 35° in 10 ml containing 1000 μ*M* Tris HCl, pH 7.4, 500 μ*M* potassium chloride, 50μ *M* magnesium acetate, 5 μ*M* GTP, 100 μ*M* PEP, 0.4 mg of pyruvate kinase, 80 μ*M* GSH, and 1.0 μ*M* hemin. A complete amino acid mixture was included containing the 19 free amino acids in the proportions described by Borsook *et al.*[13] and also including 10 μ*M* of nonradioactive L-leucine to dilute out any liberated radioactive leucine. The reaction mixture was cooled after incubation and 4.5 ml of $105,000 \times g$ re-

ticulocyte lysate supernatant were added to provide carrier hemoglobin. The ribosomes were separated by centrifugation at 105,000 × *g* for 1 hour. Of the added sRNA-bound leucine, 55 per cent was incorporated; about 20 per cent was bound to the sedimented particles and 80 per cent was in the supernatant. The supernatant only was used for analysis.

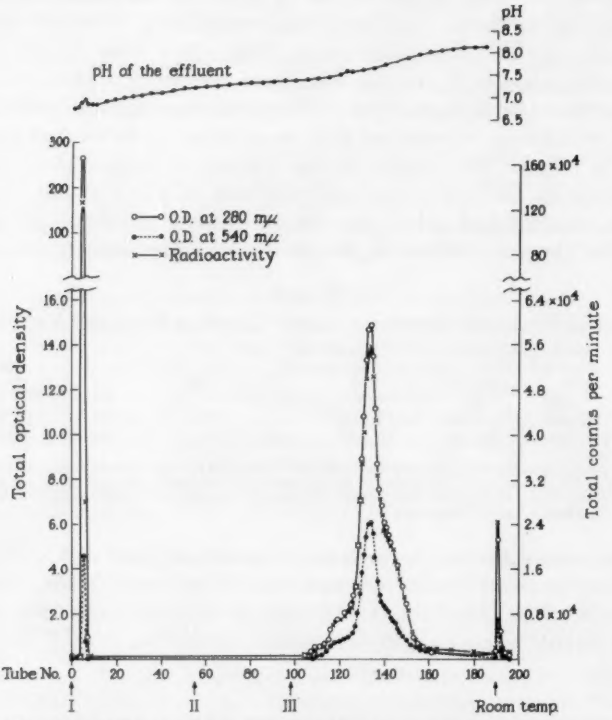

FIG. 1.—Effluent diagram of rabbit CO-hemoglobin synthesized *in vitro* by rabbit reticulocyte ribosomes and C^{14}-leucine containing *E. coli* sRNA. Conditions as described by Gutter *et al.*[14] Buffers: I. 0.01 *M* Na phosphate at pH 7.0 in 200 ml mixing chamber. Gradient to 0.01 *M* Na phosphate at pH 7.3 started immediately; II. Gradient to 0.01 *M* Na phosphate at pH 7.5; III. Gradient to 0.01 *M* Na_2HPO_4.

Hemoglobin was converted to carbon monoxyhemoglobin by blowing CO over the solution with gentle shaking for 5–10 minutes; the solution was then mixed with 0.05 vol of 1 *M* $MnCl_2$. A precipitate, comprising largely RNA, was removed by centrifugation and the supernatant dialyzed against 0.1 *M* Tris buffer, pH 7.5, containing 0.005 *M* EDTA. A small precipitate formed and was discarded. Then, the hemoglobin was precipitated by 0.9 saturation with solid ammonium sulfate, and the precipitate was dissolved in and dialyzed against 0.01 *M* sodium phosphate buffer, pH 7.0. The dialyzed solution was placed on a column of 50 × 0.9 cm CM-cellulose and eluted as noted in Figure 1. Fractions of 5–7 ml were collected

and optical densities at 280, 418, and 540 mμ, and the radioactivity were recorded for each fraction. Radioactivity distribution in the progress of purification is seen in Table 2. The fraction used for chromatography contained 57 per cent of the incorporated C^{14}-leucine. As seen in Figure 1, 75 per cent of this radioactivity

TABLE 2

PURIFICATION OF HEMOGLOBIN CONTAINING C^{14}-LEUCINE

Fraction	Protein content (mg)	Hemoglobin content (mg)	Total radioactivity (cpm)	Specific radioactivity (cpm/mg protein)	Per cent
Incubation mixture after addition of carrier hemoglobin	235	172	1.78×10^6	7,570	100
Hemoglobin solution before column	158	112	1.01×10^6	6,400	57
First peak on column	46	6	0.126×10^6	2,740	7.1
Second peak on column	94	94	0.778×10^6	8,280	44
Third peak on column	4	2	0.032×10^6	8,000	1.8

The hemoglobin was synthesized *in vitro* by rabbit reticulocyte ribosomes and C^{14}-leucine-containing *E. coli* sRNA containing 3.24×10^6 cpm. See text for incubation mixture.

coincided with the main hemoglobin peak. No non-heme protein was present in this peak as indicated by the constant ratio of optical densities at 540, 418, and 280 mμ throughout the peak, the ratios being 540 mμ/280 mμ = 0.4; 418 mμ/280 mμ = 4.5. The specific radioactivity (cpm/mg of hemoglobin) remained constant within the experimental error throughout the main peak. Of the 25 per cent of the radioactivity appearing in two minor peaks, the very early one obviously was not adsorbed. A second peak was eluted when, at the end, the column was warmed to room temperature; its specific activity was similar to that of the major peak, whereas the first peak contained a large amount of RNA and a small amount of hemoglobin mixed with non-heme protein of a lower radioactivity. The protein concentration of the early peak was determined by the Lowry method[7] because of the high optical density at 280 mμ due to the high nucleic acid content. This peak probably corresponds to the fraction designed "V" by other workers,[14, 15] and the last peak probably corresponds to the A_2-peak reported with hemoglobin from other species. Altogether, 91 per cent of the total protein on the column was recovered, and 93 per cent of the radioactivity.

The hemoglobin in tubes 128–140 was concentrated by the addition of ammonium sulfate to 0.9 saturation, redissolved in a small amount of water, and dialyzed against water for 2 days. The dialyzed hemoglobin was digested with trypsin and the peptides separated by the method of Ingram.[16] A 2-ml solution containing 18 mg of labeled hemoglobin was brought to pH 8.24 with dilute NaOH in an autotitrator and denatured in salt free solution by heating in a water bath at 90° for 6 minutes. It was cooled immediately and stirred in an atmosphere of nitrogen at 38°; trypsin, 0.02 ml of a 0.5 per cent solution in 0.001 *M* HCl was added, and the pH kept at 8.2 by automatic addition of 0.1 *N* NaOH. After 65 minutes, a further aliquot of trypsin was added and the digestion continued for an additional 40 minutes. The reaction was stopped by adjusting the pH to 6.5 with 1 *N* HCl and the precipitate was removed by centrifugation. Of the original solution, 0.2 ml was dried *in vacuo* over sulfuric acid and soda lime, dissolved in 0.020 ml of water, and the peptides separated on Whatman No. 3 MM paper cut as described by Ingram.[16]

Electrophoresis was carried out in one dimension between polished glass plates

resting on the two buffer vessels, using pyridine/glacial acetic acid/water in the proportion 10:0.4:90 by volume, at pH 6.4. A potential of 19V/cm was applied for 150 minutes. The paper was dried, and ascending chromatography in the second dimension carried out for 15 hours in *n*-butanol/glacial acetic acid/water, 3:1:1 by volume. The paper was dried again and the peptide spots were developed by dipping the paper into 0.25 per cent ninhydrin in acetone, allowing the color

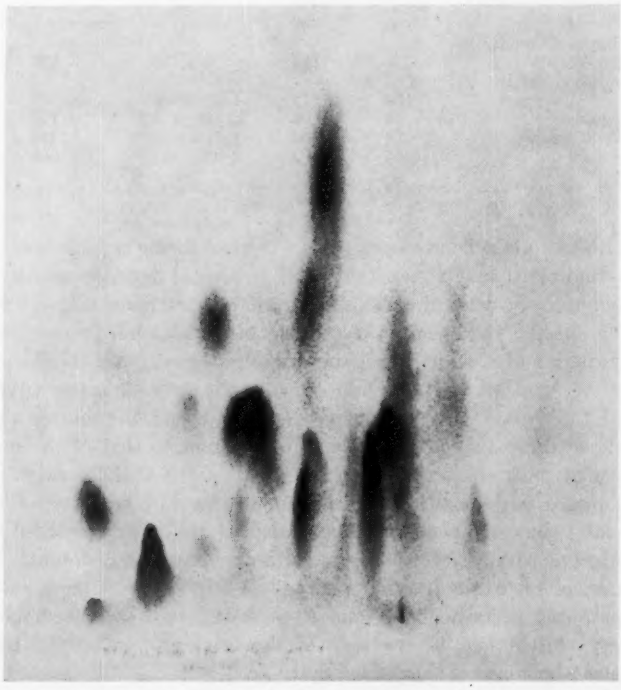

FIG. 2.—Ninhydrin stain of the fingerprint of rabbit hemoglobin obtained from tube 128–140 of the chromatogram shown in Fig. 1. See text for experimental conditions.

development to take place at room temperature. A photograph was taken after 24 hours when maximum intensity was reached (Fig. 2).

A radioautogram was made by exposing X-ray film for 10 weeks to the one-dimensional electropherogram and to the "fingerprint." In the two-dimensional pherogram, or fingerprint, 19 labeled peptides can be seen (Fig. 3). All labeled peptides are superimposable upon the authentic ninhydrin-positive peptides of the nonlabeled carrier hemoglobin. Too little is known with certainty about the structure of these peptides from rabbit hemoglobin, as, for example, whether the two more heavily labeled peptides contain two or more leucine residues per peptide, to warrant a further evaluation of these data. It seems safe, however, to conclude from the labeling pattern, by comparing it with data on human hemoglobin, that both the α and β chains of the rabbit hemoglobin are synthesized from the amino acids bound

to *E. coli* sRNA. The fact that the nonlabeled free leucine present in large amounts in the incubation mixture did not dilute out the C^{14}-leucine sRNA, makes us feel sure that the RNA-bound C^{14}-leucine was indeed incorporated and excludes incorporation by way of reactivation of free C^{14}-leucine liberated by hydrolysis of

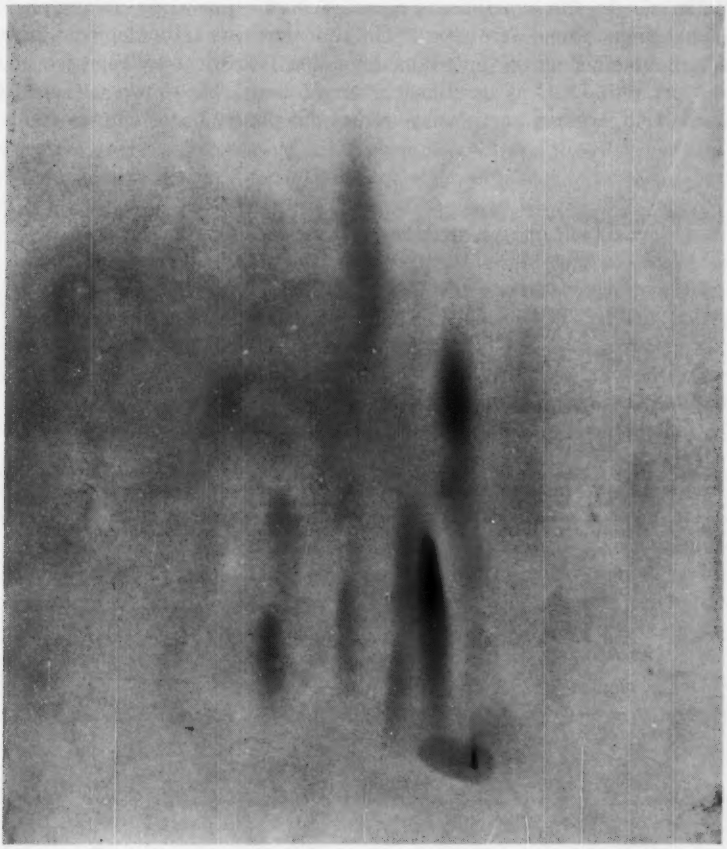

FIG. 3.—Radioautogram obtained by exposing the fingerprint shown in Fig. 2 to X-ray film for 10 weeks.

the *E. coli* aminoacyl-sRNA, which is unlikely anyway in view of the absence of ATP.

PRELIMINARY ANALYSIS OF THE COMPONENTS OF THE TRANSFER SYSTEM

During the last few years, a considerable amount of effort has been directed towards characterizing the components of the system forming peptide bonds with aminoacyl sRNA as the immediate substrate. Nathans has reported on the isolation of enzyme fractions which catalyzed the transfer from aminoacyl-sRNA to liver ribosomes[1] or *E. coli* ribosomes.[2] In order to obtain a good assay for the transfer factor, it was necessary to treat the liver particles with 0.5 per cent DOC

to remove the membrane fraction. The factor is strongly absorbed to the cruder microsomal fraction spun down at 105,000 × *g*. The same is true for a similar reticulocyte fraction. Washing three times with Medium A does not remove the factor and generally no difference could be found with and without addition of supernatant.[17] In the experiments reported in the preceding paragraphs, such microsomal preparations were used. The reticulocytes can be depleted, however, in the same manner as the microsomal fraction from liver by repeated washing with 0.5 per cent DOC as described under *Methods*. As shown in Table 3 and Fig. 4, the DOC-washed particles are nearly completely dependent on addition of

TABLE 3

COFACTOR REQUIREMENT OF AMINO ACID TRANSFER FROM *E. coli* sRNA TO HEMOGLOBIN BY RABBIT RETICULOCYTE DOC PARTICLES

Conditions	C^{14}-leucine incorporated cpm
Complete system	6,110
−PEP, kinase, GTP	122
−GTP	1,530
−PEP, kinase	3,042
−GSH	5,010
−Supernatant factor	856
+1 μM chloramphenicol	5,950
+0.5 μM puromycin	232

The reaction mixture consisted of: 0.25 μmoles of GTP, 10 μmoles of PEP, 40 μg of pyruvate kinase, 8 μmoles of GSH, 0.1 μmole of hemin, 50 μmoles of KCl, 5 μmoles of $MgCl_2$, 100 μmoles of Tris HCl buffer pH 7.4, a balanced mixture of all amino acids[18] including 1 μM of C^{12}-leucine, 2 mg of DOC particles, 0.1 mg of purified supernatant factor, and 77 μg of *E. coli* sRNA containing all amino acids including C^{14}-leucine, 10,000 cpm (specific activity 66,300 cpm/1 mμM leucine) in a total volume of 1 ml. The incubation was for 15 minutes at 37°.

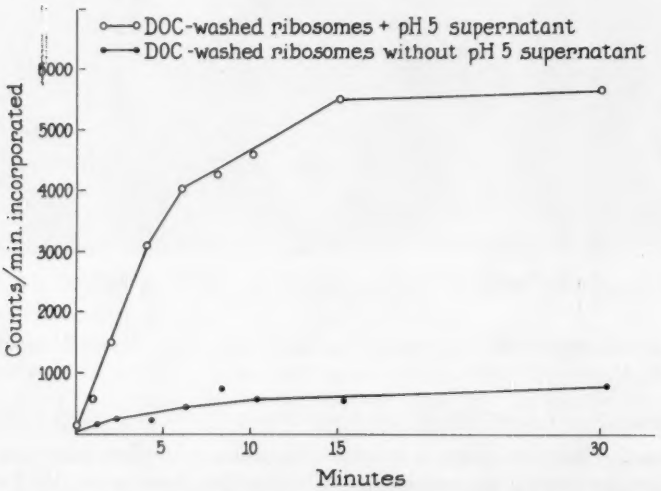

FIG. 4.—Time curve of C^{14}-leucine transfer from *E. coli* sRNA to hemoglobin. Two tubes containing 10 ml of the reaction mixture as given in Table 3 were incubated at 37°. The pH 5 supernatant from reticulocytes was used as the source of transfer factor in one tube while the other tube did not contain any transfer factor. At each time point, 1.0 ml was pipetted into 5 per cent TCA and the precipitate treated as described under *Methods*.

the supernatant and require the usual cofactors for full activity. It is of interest that the rat liver supernatant fraction could be used in place of the comparable reticulocyte fraction. The transfer factor from reticulocytes was partially purified; the pH of the 105,000 × *g* supernatant was adjusted to 5.2 and the factor precipitated from the supernatant by 0.55 saturation with ammonium sulfate followed by passage through a short column of CM-cellulose. A further purification may be obtained following roughly the procedure of Nathans.[2, 17] Recently, Bishop and Schweet[18] also reported a similar transfer factor.

Comments.—The result of this study has rather encouraged us to believe that in the system used here, consisting of aminoacyl-sRNA and the ribosomal fraction, we are dealing with the polymerization mechanism in hemoglobin synthesis. The fact that hemoglobin can be synthesized from the amino-acyl-sRNA derived from a microorganism, *E. coli*, makes us believe more strongly in the generality of what one now considers as the amino acid code represented in the amino acid specific sRNA. Comparing the components of this system with the analogous one obtained from *E. coli*, described in a recent paper,[2] GTP and a soluble enzymatic fraction are shown to be needed and both systems are inhibited by puromycin. The resistance of mammalian systems to chloramphenicol, however, in contrast to the inhibition of the analogous microbial system, is quite noteworthy. On no occasion have we observed a reliable effect of chloramphenicol with a mammalian system, even at concentrations of 0.01 molar. Differences between the mechanisms of chloramphenicol and puromycin interference have emerged from experiments with the *E. coli* system. These suggest that chloramphenicol blocks a partial reaction preceding that blocked by puromycin.[19] We are beginning to feel that the difference between the mammalian system and the microbial system must indicate a special, probably additional, feature in the microbial system which precedes the polymerization of amino acids, the mechanism of which we presently presume to be the same in all cells.

We should like to thank Dr. V. M. Ingram for introducing us to the method of fingerprinting.

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† Helen Hay Whitney Foundation Fellow.

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"REVERSIBLE" DNA

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By chemical modification of DNA, we have produced materials whose helix-coil transitions are totally reversible. This communication concerns the properties of this "reversible" DNA, which we have made by two distinctly different processes: (1) Reaction with the chemical mutagen, sodium nitrite, at pH 4.2 (HNO_2-DNA), and (2) Reaction with the cytotoxic bifunctional alkylating agent, bis(β-chloroethyl)methylamine hydrochloride at pH 7.1 (HN2-DNA).

The spectrophotometric and density-gradient centrifugation experiments reported below provide evidence that the chemical reactions leading to these products need not substantially change the secondary structure of double-helical, native DNA. This suggests the hypothesis that the reversibility of denaturation in these chemically modified DNA's is controlled by covalent bonds which link complementary strands of the double helix.

Materials and Methods.—The DNA used in these experiments was isolated from salmon testes by the method of Simmons.[1] *Pseudomonas fluorescens* DNA, used as a density reference in CsCl density-gradient centrifugation, was prepared by a method to be described elsewhere.[2]

Ultraviolet absorbance measurements were made in the following manner. A DNA solution, in a stoppered cuvette, is heated to a given temperature in the thermostated cell compartment of a spectrophotometer and the absorbance (A) at 259 mμ is measured after equilibrium is attained. The cuvette is then removed, plunged into ice to insure rapid cooling (quenching), re-equilibrated in a second spectrophotometer maintained at 25°C, and the absorbance is measured again. This heating and quenching cycle is now repeated at successively higher temperatures. Although an entire denaturation curve can be performed on a single sample, a certain saving of time is achieved by using two cells simultaneously. Measurements at a series of temperatures yield the curves shown in Figures 1 and 3. Absorbances measured at the elevated temperatures yield the curves marked (d), while measurements, at 25°C, on quenched solutions yield the curves marked (i). Appropriate control measurements are made to verify that the properties of DNA at the highest temperatures are not substantially affected by previous cycles of heating.

Preparation of HNO_2-DNA: Salmon DNA (0.1%) in 0.01 M NaCl was mixed with 4 volumes of 1.25 M $NaNO_2$, 0.3 M Na acetate, pH 4.20, and equilibrated at 25°C. After a specified reaction time, the mixture was placed in ice and 0.5 volumes 2 M Na_2HPO_4 added to neutralize (to pH 6.5 approx.). There followed 24 to 48 hours' dialysis against several changes of 0.01 M NaCl at 1°C.

Preparation of HN2-DNA: HN2, DNA in 0.01 M NaCl and phosphate buffer were mixed to give: 4.3 μ moles DNA-P, 2.4 μ moles HN2, 0.07 M phosphate pH 7.1, total volume 7.7 ml. After 45 minutes' incubation at 25°C, the mixture was cooled in ice and 1 ml added to 9 ml 8.0 M $NaClO_4$, pH 7.05 for the thermal denaturation experiment, shown in Figure 3.

Results and Discussion.—The reversibility of the thermal denaturation of HNO_2-

DNA was demonstrated in three ways—spectrophotometrically, by viscosity measurements, and by equilibrium centrifugation in a CsCl density gradient. Results of the spectrophotometric experiments are shown in Figure 1. Two types of measurement of the temperature dependence of absorbance in low ionic strength, 51 vol. per cent methanol are represented there: those made at the ambient temperature and those made on solutions which have been heated to a given temperature, rapidly cooled (quenched) in ice, and re-equilibrated at 25°C. The two curves marked O show the results for unreacted salmon DNA: It will be noted that, in this solvent, irreversibly denatured DNA is almost completely hyperchromic, and that its absorbance increases only very slightly between 25 and 55°C. Conse-

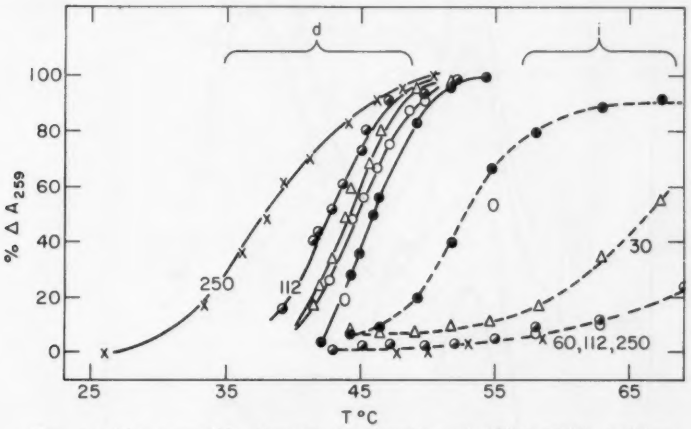

FIG. 1.—The reversible thermal denaturation of HNO_2-DNA (salmon testis) in 51% (v/v) methanol ($10^{-3}M$ NaCl, $10^{-3}M$ Tris-hydroxymethyl-aminomethane, pH 7.1).

$\%\Delta A_{259}$: Per cent of maximum absorbance increase (referred to measurement at the ambient temperature), uncorrected for thermal expansion of solvent.

$$\%\Delta A_{259} = \frac{100[A(T) - A(25°C)]_{d \text{ or } i}}{[\Delta A_{max}]_d}$$

———, absorbance measured at temperature shown, (*d*).

– – – – –, absorbance measured at 25°C on samples previously equilibrated at the temperature shown and quenched in ice, (*i*).

Time of reaction with HNO_2, pH 4.2, 25°C: zero time control, ●; 30 minutes, △; 60 minutes, ○; 112 minutes, ◑; and 250 minutes, ×.

quently, reversible changes of absorbance upon heating and cooling can be unequivocally interpreted in terms of reversible changes of long-range order. The other curves of Figure 1 show the results of denaturation experiments on DNA which has been treated with 1 *M* nitrite at pH 4.2. As the amino groups of adenine, guanine, and cytosine react,[3] the thermal stability of the DNA is decreased. This is entirely in keeping with the fact that the amino groups are involved in interactions which stabilize the secondary structure of DNA. Maximum absorbance increases on thermal denaturation, 100 $\Delta A_{max}/A(25°C.)$, are 44, 43, 40, 39, and 39 (±2) per cent, respectively, for DNA's that have been reacted for 0, 30, 60, 112, and 250 minutes. With regard to the irreversible component of denaturation, on the other hand, one soon observes a drastic change. DNA which has been reacted with nitrous

acid for more than one hour cannot be irreversibly denatured at 60°C which, in this medium, suffices to denature untreated DNA completely. Similar results are obtained when the denaturation is followed in aqueous 7.2 *M* $NaClO_4$, or 0.01 *M* NaCl.

When 70-minute-reacted HNO_2-DNA is heated in 0.01 *M* NaCl at 100°C for 15 minutes and quenched in ice, the specific viscosity (zero shear, measured

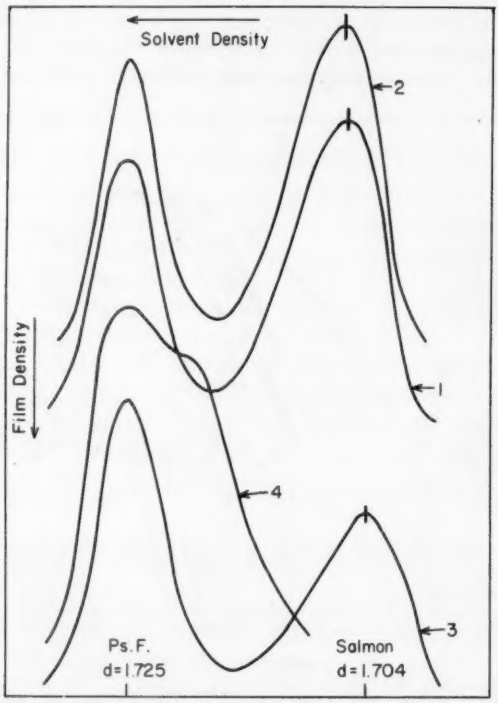

FIG. 2.—Equilibrium centrifugation of normal and HNO_2-treated DNA (salmon) in a CsCl density gradient (7.7 molal CsCl, 0.01 *M* Tris, pH 8; 40 hours centrifugation at 44,770 rpm, 25°C). DNA from *Pseudomonas fluorescens* is used as a density marker in all four experiments. Data are presented in terms of densitometric traces of absorption camera pictures through the region of the DNA "bands." Plate density baselines are displaced for ease of comparison. The buoyant densities of native salmon and Pseudomonas DNA are taken from the data of Rolfe and Meselson,[5] Sueoka, Marmur, and Doty.[4] *Curve 1*, HNO_2-DNA (70-minutes reaction); *curve 2*, HNO_2-DNA heated for 15 minutes at 100°C in 0.01 *M* NaCl and quenched; *curve 3*, unreacted DNA; and curve 4, same, but heated for 15 minutes at 100°C in 0.01 *M* NaCl, and quenched.

at 25°C) decreases only 13 per cent. Unreacted DNA subjected to the same heating and quenching cycle decreases its specific viscosity by a factor of ten.

CsCl density-gradient centrifugation of heated and unheated, reacted and control DNA, demonstrates this reversibility in yet a third way (Fig. 2). The effective densities of heated and unheated HNO_2-DNA differ by less than 0.001 gm/cm^3 and the bandwidths are almost the same (curves 1 and 2). The latter result is in keeping with the slight viscosity change upon heating, and the fact that for salmon DNA, which is relatively heterogeneous with respect to the base composition of

individual molecules, only a part of the observed bandwidth is due to Brownian motion. Curves 3 and 4 show the familiar behavior of untreated DNA in which heating to 100°C results in irreversible denaturation and its concomitant increased effective density.

Preliminary results on the kinetics of the reaction of nitrite with DNA amino groups have been reported.[6] On the basis of these results one would estimate that in one hour at 25°C, pH 4.2, less than 2 per cent of the amino groups of DNA have reacted. The complete modification of such a fundamental property of DNA as its irreversibility of denaturation at such a low extent of reaction is remarkable. In conjunction with current work on the factors controlling the irreversibility of denaturation, it strongly suggests the possibility that covalent crosslinks between

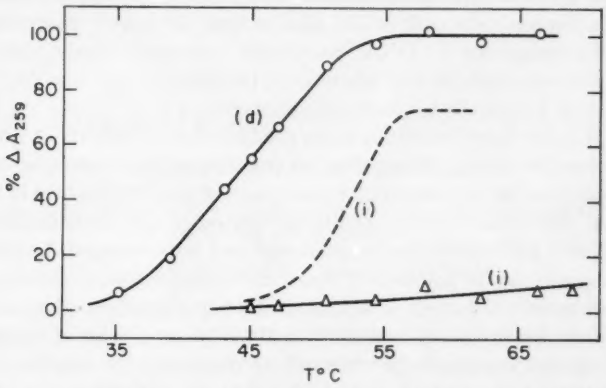

FIG. 3.—The reversible thermal denaturation of HN2-DNA in 7.2 *M* $NaClO_4$, pH 7. $\%\Delta A_{259}$, percent of maximum absorbance increase (referred to measurement at the ambient temperature) uncorrected for thermal expansion of solvent. O, absorbance measured at temperature shown, (*d*). Δ, absorbance measured at 25°C on samples previously equilibrated at the temperature shown and quenched in ice, (*i*). ———, (*i*) curve of untreated DNA, in this medium.

Comparison of properties of HN2-DNA and untreated DNA in this medium: (*a*) midpoint denaturation temperature, measured at ambient temperature ($T_{1/2,d}$), 44.1 and 44.2°C, respectively; (*b*) maximum absorbance increase upon thermal denaturation, (uncorrected for solvent expansion) 34 and 36%, respectively.

polynucleotide chains are being formed. We have, therefore, turned to a reagent whose crosslinking ability was suggested a number of years ago, namely the bifunctional nitrogen mustard, HN2.[7] In fact, we have found HN2 to be capable of converting DNA into a reversibly denaturable form (Fig. 3).

Our original interest in "reversible" DNA stemmed from the light that it casts on the mechanism of denaturation. The following interpretation, consistent with the reversibility of partial denaturation in unmodified DNA,[8] may be offered for the properties of "reversible" DNA: Wherever covalent bonds are formed between complementary chains, the contiguous base pairs are constrained to remain in a small-volume element, even when the secondary structure is completely disordered. Let the environmental conditions once more become thermodynamically favorable to the re-establishment of the ordered structure, and those nucleotides, which have been constrained by a covalent crosslink, re-associate in the "correct" conjugate sequence. By so doing, they establish the registration of the rest of the

two polynucleotide strands, and also provide a nucleus, from the ends of which the reformation of the specifically paired, double helix may be propagated. Similar considerations appear to apply to the control of denaturation reversibility in globular[9] and fibrous[10] proteins. Undoubtedly, as the folding of the internal structure becomes simpler, the ability of crosslinks to control the reversal of denaturation becomes greater, and from this point of view, the "efficiency" of DNA crosslinks should be very great indeed.

It should be made clear that the reversible denaturation of HNO_2- and HN2-"reversible" DNA on the one hand, and the "re-naturation" described by Marmur, Lane, Doty, Eigner, and Schildkraut,[11, 12] on the other, are distinctly different phenomena: (1) "Reversible" DNA can be made from heterogeneous animal sources, and not merely from bacterial or viral DNA; (2) the reversibility can be displayed in low-ionic-strength media, such as that of Figure 1, in which completely denatured bacteriophage T2 DNA "re-natures" extremely slowly, if at all,[8] as well as in high-ionic-strength media, where both processes occur; and (3) "reversible" DNA cannot be kinetically blocked (quenched) near 0°C.

Finally, it is necessary to comment on the fact that the ability of nitrous acid to produce reversible DNA, presumably by crosslinking, has not been noted before. It is probable that the intramolecular condensation products formed in this reaction are unstable under the rigorous conditions previously used for isolating and identifying RNA and DNA deamination products[6] and have escaped detection for that reason. It should also be pointed out that if the interpretation of the structural basis of DNA reversibility is correct, a very small number of crosslinks should suffice to endow a DNA molecule with reversibility, so that the acquisition of this property becomes a most sensitive assay of a variety of, as yet unspecified, reaction products.

The question of the extent to which chemical mutagenic experiments on TMV, DNA, and bacteriophage, utilizing nitrous acid,[13–16] need re-evaluation depends on a determination of the relative frequencies of crosslinking and other reactions. It seems reasonable to anticipate that crosslinking would produce entirely different modifications of genetic material from those ascribed to base transformations, perhaps leading to inactivation of sections of the phage genome rather than point mutations. The nature of these inactivations probably differs in single-stranded and double-stranded polynucleotides. In the single-stranded polynucleotides, the compact, partly ordered configuration existing at the high ionic strength of the nitrous acid reaction, would permit covalent bonds to join distant elements of the nucleotide *sequence*, whereas in native DNA, crosslinks would form between complementary strands. Perhaps, the demonstration of the chemical and physical effects of nitrous acid described in this communication accounts, in part, for the high lethality of this *in vitro* mutagen.

I should like to express my thanks to B. S. Strauss for most helpful discussions, and to W. Bloom, R. B. Uretz, and R. E. Zirkle for their comments on the manuscript. This research was supported by U. S. Public Health Service Grant C5007.

Note added in proof: I should like to direct attention to the paper of Marmur and Grossman [these PROCEEDINGS, **47**, 778 (1961)] which presents convincing evidence that a similar type of "reversible" DNA formation occurs as the result of irradiation with ultraviolet light.

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*ENZYMATIC PHOSPHORYLATION OF N-ACETYL-D-MANNOSAMINE**

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Communicated by Robert C. Elderfield, May 18, 1961

We have recently reported that D-mannosamine (2-deoxy-2-amino-D-mannose) occurs naturally in the form of its N-acetyl and N-glycolyl derivatives. These derivatives have been demonstrated in the following systems: (*a*) the reversible enzymatic cleavage of N-acetyl- or N-glycolylneuraminic (sialic) acids by NANal-dolase yields pyruvate and the corresponding N-acyl-D-mannosamine;[1] (*b*) rat liver extracts catalyze the conversion of UDP-N-acetyl-D-glucosamine to UDP and N-acetyl-D-mannosamine;[2] and (*c*) an epimerase, from *Aerobacter cloacae*, catalyzes the interconversion of N-acetyl-D-glucosamine 6-phosphate and N-acetyl-D-mannos-amine 6-phosphate.[3]

While the epimerase is present in many bacterial species grown on ordinary media, the concentration of enzyme is particularly high when the cells are grown on N-acetyl-D-mannosamine as the sole source of carbon. These data suggest that the first reaction in the metabolism of this sugar involves phosphorylation. A kinase was, in fact, detected in extracts of *Aerobacter cloacae* and a variety of animal tissues. Owing to the lability of the bacterial kinase, extracts from animal tissues were em-ployed for purification studies.

As indicated below, a specific N-acyl-D-mannosamine kinase was isolated from rat liver and catalyzed the reaction: N-acetyl-D-mannosamine + ATP → ADP + N-acetyl-D-mannosamine 6-phosphate.

Enzyme Purification.—All operations were conducted at 0° to 4° and centrifuga-tions at 24,000 × *g*. Rat liver was ground with three volumes of water in a chilled mortar with the aid of fine glass beads. After centrifugation for 30 minutes, the residue was discarded. Thirty ml of the supernatant fluid (crude extract) were treated with 0.2 volume of a 2 per cent protamine sulfate solution, the mixture stirred for 10 minutes, and centrifuged for 10 minutes. The resulting precipitate,

containing the enzyme, was washed with 12 ml of water, and successively extracted with 12 ml portions of phosphate buffers, pH 7.5, of increasing molarity. Most of the kinase activity appeared in the 0.04 *M* and 0.05 *M* extracts, which were kept separate and further fractionated with ammonium sulfate by conventional methods.

The results of the purification procedure are shown in Table 1. Since rat liver is known to contain a kinase for N-acetyl-D-glucosamine,[4] the activities of the fractions with this substrate were determined and are shown in the table. The data clearly indicate that the two acetylhexosamines are phosphorylated by different kinases.

TABLE 1

PURIFICATION OF N-ACETYLMANNOSAMINE KINASE FROM RAT LIVER

Fraction	Specific Activity*		Yield N-AcMm kinase
	N-AcGm	N-AcMm	%
Crude extract	0.21	0.14	100
Ex I; 0.04 *M* phosph.	0.63	1.8	38
Ex II; 0.05 *M* phosph.	6.7	4.9	59
Ex-I; 0–33% amm. sulf.	0.01	4.8	27
Ex-I; 33–50% amm. sulf.	0.76	0.46	6
Ex-II; 0–33% amm. sulf.	2.1	20	26
Ex-II; 33–50% amm. sulf.	25	4.0	6

* The specific activities of the different fractions were followed with two substrates, N-acetyl-D-mannosamine (N-AcMm) and N-acetyl-D-glucosamine (N-AcGm). The assay mixtures contained the following (in μmoles) in a final volume of 0.325 ml.: ATP, 5.0; $MgCl_2$, 5.0; tris buffer, pH 7.5, 12.5; acetylhexosamine, 0.75; and the enzyme fraction. After incubating at 37° for 30 minutes, the reaction was stopped with $Ba(OH)_2$ and $ZnSO_4$ in the conventional manner, and the supernatant fluid was assayed for residual acetylhexosamine substrate by the Morgan-Elson color reaction. The specific activity is defined as the μmoles of substrate phosphorylated per mg of protein under the assay conditions described above.

Specificity studies with the partially purified fractions demonstrated that N-acetyl-D-mannosamine kinase is distinct and separable from the kinases which phosphorylate the following sugars: D-glucose, D-mannose, N-acetyl-D-glucosamine, and N-acetyl-D-galactosamine. On the other hand, the partially purified preparations exhibited approximately the same activity with N-glycolyl-D-mannosamine as with N-acetyl-D-mannosamine. The possible significance of this observation is discussed below.

Identification of Product.—In addition to ADP, the product of enzyme action is suggested to be N-acetyl-D-mannosamine 6-phosphate. This conclusion is based upon the following data.

In a large-scale preparation, 1 mmole of N-acetyl-D-mannosamine[5] was treated with enzyme and ATP under the conditions described in Table 1. Assay of an aliquot of the reaction mixture following 10 hours of incubation showed that approximately 750 μmoles of substrate were phosphorylated. The mixture was adjusted to pH 4.5, deproteinized by heating at 100° for 2 minutes, and the supernatant fluid treated with charcoal (Darco G-60) to remove nucleotides. Inorganic phosphate was removed with barium hydroxide, and the product was further purified by ion-exchange techniques as described for other N-acetylhexosamine 6-phosphates.[6] Finally, the acetylhexosamine phosphate peak was pooled and converted to its barium salt.

The isolated product was compared with synthetic N-acetyl-D-mannosamine 6-phosphate;[7] the two compounds exhibited identical properties. Thus the ratio of N-acetylmannosamine to organic phosphate in the biosynthetic product was 1.06 to 1.00. The phosphate group exhibited the expected acid stability. Paper

electrophoresis of the compound in 0.05 *M* borate buffer, pH 9.5, and in the 0.05 *M* phosphate buffer, pH 7.3, indicated its homogeneity and that it migrated at the same rate as the authentic material. The optical rotation, $[\alpha]_D^{23}$, of the product was +11.6°, and of the synthetic material +11.2° (both concentrations were 1% in water calculated as the free acid). Treatment of the phosphate ester with potato phosphatase yielded inorganic phosphate and an acetylhexosamine identified by paper chromatography and paper electrophoresis as N-acetylmannosamine.[5]

The position of the phosphate group was established by periodate oxidation using conditions previously described for other hexosamine phosphates.[6] The phosphate ester resulting from periodate oxidation was isolated by ion-exchange chromatography[8] and characterized as glycolaldehyde phosphate by its specific color reaction[9] and by its rate of migration on paper chromatography where it appeared identical with an authentic sample kindly supplied by Clinton Ballou.[10] Glycolaldehyde phosphate could only have resulted if the product of enzyme action contained the phosphate group attached at the C-6 position.

Finally, the product was shown to be N-acetyl-D-mannosamine 6-phosphate on the basis of the fact that it can completely substitute for the synthetic material in the enzymatic reaction described in the accompanying paper, i.e., in the formation of N-acetylneuraminic acid 9-phosphate.

Comments.—The data presented above indicate the presence in rat liver of a kinase for N-acetyl-D-mannosamine, the product being the corresponding 6-phosphate ester. This enzyme is distinct from well recognized hexo- or glucokinase, and from the kinases which act upon N-acetyl-D-glucosamine and N-acetyl-D-galactosamine. In a recent communication[11] concerned with the enzymatic synthesis of N-acetylneuraminic acid, Warren and Felsenfeld indicated that their fraction II acted on N-acetyl-D-mannosamine and ATP to yield a product that served as an intermediate in the synthesis of N-acetylneuraminic acid. Their enzyme is apparently the same as the kinase reported here.

The physiological significance of the kinase reaction is of considerable interest. As shown in the accompanying paper, N-acetyl-D-mannosamine 6-phosphate is a key intermediate in the biosynthesis of N-acetylneuraminic acid.

Finally, the action of the partially purified kinase on N-glycolyl-D-mannosamine should be noted. Whether the kinase described here is a single protein which acts both on the N-acetyl- and N-glycolyl-D-mannosamine or is a mixture of two enzymes is not yet known. If a specific kinase exists for the N-glycolyl derivative, this could account for the variation in the types of sialic acids (i.e., N-acetyl and N-glycolyl) between species of animals and between different organs in the same animal.[12]

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† Post-doctoral Fellow, The Helen Hay Whitney Foundation.

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*ENZYMATIC SYNTHESIS OF SIALIC ACID 9-PHOSPHATES**

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We have previously reported[1] the isolation of a specific aldolase (NANaldolase) which reversibly cleaves two of the sialic acids as follows (K_{eq}, 0.1 *M*): N-acylneuraminic acid $\rightleftharpoons$ N-acyl-D-mannosamine + pyruvate. While the enzyme catalyzes the synthesis of N-acetyl and N-glycolyl sialic acids, at low substrate concentrations the equilibrium favors cleavage. Further, despite the wide distribution of NANaldolase in animal tissues,[2] we have been unable to detect significant activity in those tissues that secrete sialic acid containing mucins. For these reasons, alternate pathways were sought for the biosynthesis of the neuraminic acids.

Such a pathway has recently been reported by Warren and Felsenfeld.[3] These authors demonstrated the synthesis of N-acetylneuraminic acid (NAN) by protein fractions obtained from rat liver when incubated with N-acetyl-D-mannosamine, PEP, ATP, Mg, DPN, and TPN. Although free NAN was isolated, the data indicated that: (*a*) NANaldolase was not involved; (*b*) several steps were probably required for the over-all reaction; (*c*) one step appeared to be the phosphorylation of N-acetyl-D-mannosamine. The accompanying paper describes the data on the kinase reaction obtained in this laboratory; the product is N-acetyl-D-mannosamine 6-phosphate.

It appeared possible that one of the steps in the new system might be a condensation reaction between PEP and either N-acetyl-D-mannosamine or the corresponding 6-phosphate ester. We had previously considered such a condensation but failed to demonstrate it.[1] Our experiments had been based on the analogy in structure between the sialic acids (2-keto-3-deoxy-nonulosaminic acids), and the known 2-keto-3-deoxy-heptonic and -octonic acids. The latter compounds are enzymatically formed by condensation of PEP with the corresponding tetrose- or pentose-phosphate.[4, 5]

A re-examination of our previous experiments, using the mild homogenization techniques emphasized by Warren and Felsenfeld,[3] has now led to the isolation of an enzyme from pig submaxillary gland extracts which catalyzes the following

reaction: N-acyl-D-mannosamine 6-phosphate + phosphoenol pyruvate (PEP) → N-acylneuraminic acid 9-phosphate + inorganic phosphate (Pi). The name N-acylneuraminic acid 9-phosphate synthetase is tentatively proposed for this enzyme.

The isolation of free NAN in the rat liver system[3] indicates the probable presence of contaminating phosphatase in those preparations; indeed, since the phosphorylated substrates were quantitatively converted to free NAN, the existence of a specific NAN 9-P phosphatase is suggested.

Specificity.—The enzyme was purified approximately 130-fold from pig submaxillary gland extracts; the final preparation exhibited a specific activity of 12 μmoles of product formed per hour per mg of protein. When the enzyme was incubated with PEP and Mg, only two sugars were found to serve as acceptors of the three carbon unit, N-acetyl-D-mannosamine 6-phosphate and N-glycolyl-D-mannosamine 6-phosphate.[6] The following compounds were inactive: N-acetyl-D-mannosamine, N-glycolyl-D-mannosamine, either sugar in the presence of ATP, and N-acetyl-D-glucosamine 6-phosphate. Pyruvate and oxalacetate would not substitute for PEP even when supplemented with ATP. Finally, DPN and TPN did not stimulate the reaction, nor did they permit substitution of N-acetyl-D-mannosamine for the corresponding 6-phosphate ester.

Stoichiometry studies with the purified enzyme showed that incubation of the enzyme with 1.0 μmole of each substrate, and stopping the reaction before completion, led to the disappearance of 0.60 μmole of N-acetyl-D-mannosamine-6-P and the formation of 0.58 μmole of NAN-9-P. The balance studies with PEP and inorganic phosphate are not yet completely satisfactory due to the presence of a contaminating phosphatase which acts on the PEP.

The purified enzyme from pig submaxillary gland utilized both N-acetyl and N-glycolylmannosamine 6-phosphate, indicating the mode of formation of the two corresponding sialic acids. As pig submaxillary mucin contains both types of sialic acid, conceivably two enzymes were present in the purified fraction, each specific for either the N-acetyl or N-glycolyl derivative. This hypothesis was tested by partial fractionation and assay of the enzyme from sheep submaxillary gland (sheep mucin contains only NAN).[7] However, the extract showed similar activity with both substrates. While the question therefore remains unresolved at this time, the available data lead to the supposition that a single synthetase acts on both compounds.

Characterization of Product.—In a large-scale incubation, 300 μmoles of C^{14}-acetyl labeled N-acetyl-D-mannosamine 6-phosphate were treated with 300 μmoles of PEP in the presence of 1.5 mg of the purified enzyme preparation, 1 mmole of Tris buffer, pH 7.2, and 0.7 mmole of $MgCl_2$. After 7 hours' incubation, the thiobarbituric acid assay[8] indicated the formation of 120 μmoles of product. The reaction mixture was placed on 100 ml of Dowex-1, formate resin and fractionated with a linear gradient consisting of 500 ml of 0.4 *M* formic acid in the mixing chamber and 500 ml of 0.4 *M* formic acid, 0.4 *M* ammonium formate in the reservoir. No significant quantity of free NAN was eluted from the column. Unreacted substrate appeared in the eluate between 300 and 400 ml, while the product was eluted between 850 to 950 ml in the form of a sharp symmetrical peak. After removal of the formic acid and ammonium formate, the product was lyophilized and isolated as its potas-

sium salt; it was contaminated with chloride ion, but contained no significant quantity of inorganic phosphate. Final purification of the compound was effected by conversion to the barium salt and precipitation from aqueous solution with ethanol yielding 65 μmoles of material. The product was characterized as NAN-9-P as described below.

Following removal of Ba^{++}, the compound exhibited the following analyses (molar ratios): nitrogen, 1.00; organic P, 0.96; inorganic P, 0.00. The specific activity of the product in terms of counts per minute per μmole of organic P was the same as that of the substrate.

A positive thiobarbituric acid color reaction was obtained with the compound with an absorption peak at 549 mμ; the extinction coefficient at the peak, under the conditions described by Aminoff,[8] was approximately 78 per cent of that obtained with free NAN. The compound also gave a positive resorcinol color reaction.[9]

Examination of the material by paper chromatography (3 solvent systems) and paper electrophoresis (pH 4.5 and 7.3) showed the substance was homogeneous, distinctly different from NAN, and that it was considerably more highly charged. For example, its electrophoretic rate of migration at pH 7.3 was 2.8 times that of free NAN.

Quantitative hydrolysis of the phosphate ester was effected with potato acid phosphatase yielding inorganic phosphate and a sugar acid. The latter was purified by ion-exchange techniques and characterized as NAN since it: (*a*) exhibited the same specific activity as the parent compound; (*b*) migrated at the same rate as authentic NAN in 3 paper chromatographic solvent systems[10] and on paper electrophoresis in borate buffer at pH 9.5; (*c*) was quantitatively cleaved by NANaldolase to pyruvate (identified with lactic dehydrogenase) and an acylhexosamine. The acylhexosamine was characterized as N-acetyl-D-mannosamine since it: (*a*) gave the typical Morgan-Elson color reaction; (*b*) exhibited the same specific activity as the parent compound; (*c*) behaved in an identical manner to the authentic compound on paper electrophoresis and chromatography. The latter procedures would clearly distinguish the acylhexosamine from N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, and the corresponding N-glycolyl derivatives of the three hexosamines.

The known specificity of NANaldolase,[1] i.e., it will not act on N-acyl-D-mannosamine 6-phosphate esters, suggested that the enzyme would not act on NAN-P. This indeed was found to be the case.

Proof that the phosphate group was attached to C-9 of the NAN moiety was obtained by periodate oxidation, using the conditions described for structural studies on hexosamine phosphates.[11] The phosphate ester formed by the periodate oxidation was characterized as glycolaldehyde phosphate by ion-exchange chromatography, its specific color reaction,[12] and by its rate of migration on paper chromatography (identical to that of an authentic sample kindly supplied by Clinton Ballou).[13]

In summary, an enzyme (N-acylneuraminic acid 9-phosphate synthetase) has been isolated from pig submaxillary glands. The enzyme catalyzes the following reaction:

N-acyl-D-mannosamine 6-phosphate + PEP →
N-acylneuraminic acid 9-phosphate + Pi.

Whether the N-acetyl- and N-glycolylneuraminic acid 9-phosphates are formed by the same enzyme remains to be established as well as the possible relationship between these substances and the biosynthesis of nucleotides containing sialic acids.[14]

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*INDUCTION BY ARGININE OF ENZYMES OF ARGININE BIOSYNTHESIS IN ESCHERICHIA COLI B**

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In many cases the formation of an enzyme has been observed to be induced by its substrate and repressed by the end-product of the sequence in which it participates. Thus it appeared a paradox when the discovery of the nonrepressibility by arginine of ornithine-transcarbamylase (enzyme 5)† in *Escherichia coli* strain B[1] disclosed that arginine actually stimulates the synthesis of this enzyme.[2] This stimulatory effect is modest in wild type B, which in the presence of arginine increases the enzyme level twofold. This effect has also been observed in bacteria of other species.[3] However, we have now obtained hybrids of strain B with K12

exhibiting much increased inducibility as well as mutants of B which are no longer induced by arginine. Moreover, it has been shown that induction affects at least two enzymes of the arginine sequence, and a genetic block between these enzymes and arginine does not affect induction. It is thus clear that this newly observed feedback induction, like the already well-known feedback repression, concerns several (if not all) enzymes of the sequence and is not sequential.

An attempt has been made[4] to explain this perplexing situation in terms of current concepts about end-product repression. It was speculated that arginine may compete with the repressor (say arginine—RNA) if this latter is altered and consequently has a low affinity for its site of action. This hypothesis would imply that the low enzyme level found in strain B[2] would be due to incomplete end-product repression. However, this conclusion is contradicted by the observation that the enzyme level may not be raised by growing an arginine auxotroph of strain B in a chemostat under arginine limitation. This result and genetic evidence discussed elsewhere[5, 6] support the assumption that the arginine biosynthetic pathway in strain B is under a new kind of control, different from repression by end-product. For this second control we have provisionally suggested the term "modulation."[5]

The present paper describes arginine induction in detail and considers its possible relationship to modulation.

Bacterial strains: The following strains of *E. coli* were used in the present experiments.

Wild type B.

B4S-7, a spontaneous mutant derived from B.[5] B4S-7 is a prototroph, like B, but possesses a high level of arginine enzymes.

BP8 and BB18, hybrids between strains B and K12. BP8 was obtained by crossing B with Hfr K10, BB18 by recrossing BP8 with the same donor. Since in such crosses B and BP8 are the recipient cells, BP8 and BB18 may be considered essentially B derivatives. They are both prototrophs.

Wc2, a prototroph nonrepressible by arginine isolated by I. B. Weinstein from the repressible strain W using canavanine for selection.[7]

K12 Hfr mutants were used as donors in the recombination experiments: K10 a prototroph originally from L. Cavalli, 3000 B_1^-, originally from W. Hayes, and P76 obtained from F. Jacob. These strains differ in the time of injection of the Lac marker.‡ Auxotrophic mutants were obtained by UV irradiation and selection by penicillin with an improved method.[8] Mutant BP8-I-III with two blocks in the arginine pathway, one before ornithine and a second after citrulline, was obtained and checked as previously described.[2] The Sm^R mutants were selected on nutrient agar plates containing 500 μg/ml of streptomycin. Valine as a selective marker depended on the natural sensitivity of K12 and resistance of B.

Growth conditions: The organisms were grown with aeration at 37° (when not otherwise indicated) in mineral-citrate medium A[9] supplemented with arginine or other growth factors when necessary. Glucose or other carbon sources (when indicated) were used at 0.1%. When enriched medium was used, 0.2% casein hydrolysate (NZ-Case Sheffield) and 0.2% yeast extract (Difco) were added to the minimal medium A. The chemostat experiments followed the usual procedure.[10]

Genetic recombination experiments: Exponentially growing cultures of the mates in enriched medium (bacterial density about 10^8 cells/ml) were mixed (1 to 1) and left at 37° for 150 min (5 ml total in a 125 ml flask to insure good aeration). Then 0.1 ml portions of a 10^{-2} dilution of the mating mixture were spread on appropriate selective plates. The various selective factors were used at the following concentrations: histidine, 20 μg/ml; arginine, 100 μg/ml; streptomycin, 500 μg/ml; valine, 100 μg/ml; lactose (instead of glucose), 0.1%. A first rough screen for repressibility by arginine (R_{arg}) was done by scoring the Sm marker: 98% of recombinants carrying the Sm marker of the recipient also had its R_{arg} character; 80% of those having the Sm marker of the donor also had its R_{arg} character.[5] The final score of R_{arg}, of high or low enzyme level (Md),

and of inducibility characters was done by direct enzyme determination (generally enzyme 5 when not otherwise indicated) in cells grown in minimal glucose medium with or without arginine (100 μg/ml).

Determination of enzyme activity: Enzyme 5 or 7 activity was determined on toluenized cells as previously indicated.[6, 11] One unit of enzyme 5 or 7 is the amount which synthesizes 1 μmole of citrulline or arginine respectively per hour. The figures reported in the tables are units of enzyme per ml of culture measuring optical density 1 at 490 mμ in a Beckman spectrophotometer. Thi_8 optical density roughly corresponds to 1 mg bacteria (dry weight).

Strains Inducible by Arginine.—The effect of arginine on the synthesis of enzymes 5 and 7 has been tested on different strains not repressible by arginine (R_{arg}^-). Table 1 indicates that the strains with a low level of these enzymes were

TABLE 1

INDUCING EFFECT OF ARGININE ON ENZYMES 5 AND 7 IN DIFFERENT STRAINS NOT REPRESSIBLE BY ARGININE

Strains #	Origin	Specific Activity of Enzyme 5 Arginine during growth Absent	Enzyme 5 Arginine during growth Present	Specific Activity of Enzyme 7 Arginine during growth Absent	Enzyme 7 Arginine during growth Present
B	"Low level" wild type	5.4	10.5	0.21	0.38
BB18	Hybrid B × K12	10.6	57.8	0.14	0.48
B4S-7	"High level" B mutant	178.0	180.0	1.50	1.60
We2	Nonrepressible W mutant	89.0	92.0	0.75	0.76

The cultures were grown at 37° with aeration to exhaustion of glucose in minimal medium A with or without addition of 100 μg/ml arginine. Specific activity: enzyme units/mg dry weight bacteria.

induced to varying degrees, whereas the strains with a high level were not further stimulated by arginine. It appears that the amount of enzyme made by B4S-7 corresponds to the maximal cell capability: inducibility has been demonstrated only in cells with a low enzyme level.

Specificity of Induction.—Various substances related to arginine or to general metabolism have been tested for their ability to induce enzyme 5 synthesis. These include some of the biosynthetic precursors of arginine (citrulline, ornithine, glutamate); the end-products of related biosynthetic pathways (uracil, proline); the other basic amino acids (lysine, histidine); the product of arginine degradation usually found in these strains (agmatine); the stereoisomer analog (D-arginine); canavanine; and finally a mixture of common amino acids, nucleic acid bases, and vitamins. It can be seen in Table 2 that L-arginine and its immediate precursors were the only inducers.

Because of the reversibility of the reaction connecting ornithine to citrulline and arginine, one cannot decide whether these three compounds are independently active or whether only one of them is able to induce, the others being converted into this one by cellular metabolism. To settle this point, we isolated an arginine auxotroph from BP8 (BP8-I-III) with two blocks in the arginine pathway, one before ornithine and another after citrulline. The effect of ornithine, citrulline, and arginine was tested by growing this organism in a chemostat under steady-state limitation either by arginine (10 μg/ml) or by glucose (with arginine 50 μg/ml, leaving a calculated residue of 40 μg/ml). To the culture growing under arginine limitation 40 μg/ml of either ornithine or citrulline was added. In contrast with Table 2, Table 3 shows that these intermediates did not induce in an organism that could not convert them into arginine. The inducing effect of arginine therefore appears to be specific.

TABLE 2

INDUCING ACTIVITY OF L-ARGININE COMPARED WITH OTHER COMPOUNDS

Additions to the medium	Specific activity of enzyme 5
None	8.8
L-arginine (100 μg/ml)	40.7
Citrulline "	37.9
Ornithine "	38.5
Glutamate "	7.2
ProUracil (20 μg/ml)	12.3
Lysline (50 μg/ml)	8.5
Hisine "	7.5
Agtidine "	9.0
D-matine (100 μg/ml)	6.8
Arginine "	12.8
Canavanine (50 μg/ml)	7.5
Difco arginine assay medium	5.5
Same + arginine (100 μg/ml)	40.2

A culture of BP8 was grown at 37° with aeration to exhaustion of glucose in minimal medium A plus the additions Difco arginine assay medium contains all growth factors for *Leuconostoc mesenteroides* with the exception of arginine (see Difco Manual, 9th edition, page 235). It was used at 0.4%. Specific activity: enzyme units/mg dry weight bacteria.

It should be noted (Table 2) that in cells grown in a rich medium deprived of arginine (Difco medium) the enzyme level is lower than in those grown in minimal medium. It has been shown that arginine prototrophs in such a rich medium grow under limitation of this amino acid, and hence are derepressed in a repressible strain.[12] Therefore this result indicates that induction by arginine also responds to the endogenous arginine concentration. A similar indication has been obtained in the chemostat experiment of Table 3. It was found that at increased growth rates (division time close to 1 hr instead of 2 hr), i.e., with higher internal levels of arginine, the level of enzyme 5 tends to increase, approximating that found with arginine in excess. Thus, it appears that enzyme formation not only is stimulated by addition of exogenous arginine, but also is decreased by lowering the level of the endogenous arginine.

Influence of the Carbon Source and Growth Temperature on Basal Level and on Extent of Induction.—It is conceivable that arginine specifically counteracts a repressor produced by the general metabolism. To test this hypothesis, cells of different strains were grown in different carbon sources with or without addition of arginine. By examining the results presented in Table 4, one may deduce the following:

TABLE 3

ABSENCE OF INDUCTION BY ORNITHINE OR CITRULLINE IN DOUBLY BLOCKED BP8-I-III

Steady-state conditions	Residual arginine (μg/ml) (calculated)	Additions to chemostat (40 μg/ml)	Enzyme 5 specific activity
Growth* controlled by arginine	0	None	11.8
	0	Ornithine	10.3
	0	Citrulline	12.0
Growth† controlled by glucose	40	None	61.6

BP8-I-III is grown in chemostat at 37° and at division time = 2 hr. The growth factors per liter of medium A and the culture density are as follows:

Condition	Glucose gr.	Arginine mg	O.d. at 490 mμ
*	1.0	10	0.244
†	0.4	50	0.274

Specific activity = enzyme units/mg dry weight bacteria.

TABLE 4

INFLUENCE OF THE CARBON SOURCE ON THE BASAL ENZYME LEVEL AND ON INDUCTION BY ARGININE

Strains			Arginine in the medium	Specific Activity of Enzyme 5 in Cells Grown in			
#	Enzyme level	Induction in glucose		Glucose	Glycerol	Lactate	Succinate
B4S-7	High	Absent	Absent	184.0	202.0	180.0	—
			Present	180.0	200.0	189.0	—
Wc2	High	Absent	Absent	120.0	145.0	104.0	—
			Present	125.0	136.0	110.0	—
BB18	Low	High	Absent	13.7	100.0	15.2	62.0
			Present	59.0	103.0	55.0	87.0
B	Low	Slight	Absent	11.0	31.0	8.0	8.7
			Present	21.0	38.0	15.0	17.0
BC39	Low	Absent	Absent	3.1	10.1	4.2	9.0
			Present	3.2	23.7	4.0	17.2

The cultures were grown at 37° with aeration to exhaustion of the carbon source in minimal medium A with or without addition of 100 μg/ml or arginine. Occasional samples were also removed during the log phase and not found to be significantly different. Specific activity: enzyme units/mg dry weight bacteria.

strains like B4S-7 and Wc2 possess a high level of enzyme irrespective of the nature of the carbon source and are insensitive to arginine. In contrast, in strains inducible by arginine, such as B or BB18, the enzyme level is more or less strongly dependent upon the nature of the carbon source. The higher the inducibility, the higher the carbon source dependency. For instance, when BB18 is grown in glucose (or lactate) the basal level is low and induction by arginine is quite evident, whereas when it is grown in glycerol (or succinate) the basal level is higher and arginine is barely effective. This behavior is consistent with the idea that the high enzyme level in B4S-7 or Wc2 is the "constitutive" level and that in the inducible strains the enzyme level is under control of a repressor which originates from the general metabolism and can be counteracted by arginine. Changing the carbon source presumably reduces the rate of repressor synthesis and thus makes arginine competition more effective. For example, strain BC39, which is a recombinant B × K12 with low enzyme level (Md^+), is not inducible by arginine when grown in glucose or lactate, but when grown in glycerol or succinate the basal level is increased and further inducibility by arginine becomes evident. These results suggest that the observed noninducibility in glucose is not due to absence of sensitivity to arginine, but rather is due to excessive formation of repressor.

The effectiveness of arginine as an inducer also varies with temperature. In all inducible strains studied, a shift of temperature from 37° to 26° changes only slightly the basal level of enzyme 5 or 7 in glucose medium but remarkably increases the extent of induction. In strain BB18, for example, as shown in Table 5, at 37° arginine increased the basal level of either enzyme only 3 times, but at 26° it increased the level 14 times for enzyme 5 and 9 times for enzyme 7. It is conceivable that at 26° repressor is formed at a rate still sufficient to saturate the repressible

TABLE 5

TEMPERATURE EFFECT ON INDUCTION

Arginine in the medium	Specific Enzyme Activity of Cells Grown at 26°		Specific Enzyme Activity of Cells Grown at 37°	
	Enzyme 5	Enzyme 7	Enzyme 5	Enzyme 7
Absent	10.8	0.10	13.0	0.14
Present	149.0	0.88	51.0	0.48

Cultures of BB18 growing exponentially with aeration in minimal-glucose medium A with or without addition of 100 μg/ml of arginine. Specific activity: enzyme units/mg dry weight bacteria.

system (the basal level is unchanged) but is more efficiently counteracted by arginine.

Genetics of Inducibility.—Recombination experiments were undertaken to study the genetic relationship of arginine induction to arginine repression and to modulation. So far, this approach has had some major limitations. One is that in crosses with K12 we were able to use strain B only as a recipient; hence, all hybrids had their cytoplasm derived from B. Furthermore, inducibility could not be selected directly, and its screening required a tedious determination of enzyme activity in clones grown under different conditions; hence, only a limited number of recombinants could be analyzed.

With respect to repressibility and modulation, the mates had the following phenotype and genotype. The arginine enzymes of the K12 donor cells are repressible by arginine and are boosted to a high level under conditions where arginine limits growth. In the recipient cells, the same enzymes are not repressible by arginine, and their level is low in B and high in B4S-7. On the basis of genetic evidence discussed elsewhere,[5] repressibility by arginine (R_{arg}) and level (Modulation = Md) are controlled by two genetic factors that can be separated from each other and from the structural genes of the enzymes. They appear to be pleiotropic regulatory genes. The donors used here were $R_{arg}^{+}Md^{-}$ and the recipients either $R_{arg}^{-}Md^{+}$ (B) or $R_{arg}^{-}Md^{-}$ (B4S-7).

Phenotypic expression of inducibility can occur only in R_{arg}^{-} cells, for in R_{arg}^{+} it would be masked by repressibility. (Accordingly, in crosses yielding a mixed population of R_{arg}^{+} and R_{arg}^{-} recombinants, only the R_{arg}^{-} progeny were further analyzed.) Moreover, since it appears that the high enzyme level in Md^{-} strains corresponds to maximal use of their capacity for making that enzyme, there would be no way in Md^{-} strains, except further genetic analysis, for determining the inducibility genotype.

The results of the genetic studies establish the following points. (*a*) *Inducibility and genes concerned with enzyme structure:* To determine the relationship between inducibility and the gene for the structure of one of the enzymes affected, mutants Arg 5^{-} were obtained from B and B4S-7. (The inducibility pattern of their parents was conserved in these mutants as shown by the behavior of enzyme 7.) B Arg $5^{-}Sm^{R}$ and B4S-7 Arg $5^{-}Sm^{R}$ were crossed with Hfr K10 $Arg^{+}Sm^{S}$. Recombinants Arg^{+} were selected in the presence of Sm, and enzyme 5 level and inducibility were then determined in 50 recombinants B × K10 and 20 recombinants B4S-7 × K10. All the recombinants B4S-7 × K10 were Md^{-} and, like the original B4S-7 parent, were not further inducible. Of recombinants B × K10 96 per cent were Md^{+} and inducible like their original parent B. The remaining 4 per cent were Md^{-} and not inducible. This proves that genetic material for enzyme 5 from the donor may assume the inducibility pattern of the recipient. Moreover, by crossing B with K10 one obtains inducible recombinants with various I indexes (see Tables 6 to 8). In every case that has been analyzed, the I indexes for enzymes 5 and 7 were very similar.

These results indicate that the inducibility by arginine observed here can be separated from the genes determining the structure of the individual enzymes.

(*b*) *Inducibility and modulation:* One may analyze the experiments discussed in the preceding section from another point of view. The crosses were performed

between recipients either Md^+ (B) or Md^- (B4S-7) and the same donor Md^- (K10). It was found that inducibility was observed only with Md^+ recombinants. The inability of Md^- recombinants to be further induced was independent of the type of their recipient parent (either B inducible or B4S-7 not inducible). The same results were obtained in other similar experiments (not involving Arg^-) totalling more than 100 recombinants. This pattern is consistent with the hypothesis that induction by arginine reflects a competitive interference with a type of repression (Md) peculiar to strain B. Only cells possessing this repression (Md^+) can be induced.

To analyze further the genetic relationship between inducibility and modulation, the inducibility of enzyme 5 was studied in the 48 Md^+ recombinants obtained in the cross B × K10 discussed in the preceding section. It was found that the basal enzyme level, with but two exceptions, ranged between 3.1 and 8.9 units with an average of 5.6 units. (The two exceptional clones had a level of 11.3 and 15.5 units.) The enzyme level after growth in the presence of arginine was then determined, and the inducibility index was calculated. Table 6 shows that from the same cross, where the I index of the recipient parent was 2, one may obtain recombinants with different I indexes ranging from 1 (zero inducibility) to 7. A clone representative of each group was subcultured several times and tested again for the I index of enzymes 5 and 7. In this way, it was ascertained that the I index is inheritable and similar for both enzymes. This result indicates that inducibility has a genetic control distinct from modulation and that it is a multigenic affair.

(*c*) *Genetic factors controlling the degree of inducibility:* Some attempts were made to analyze the genetic basis for the various degrees of inducibility observed. The recombinant with the highest I index, BP8, was crossed again with the same donor, K10. The cross was analogous to the preceding one: BP8 $Sm^RVal^RHis^-$ was the recipient and Hfr K10 $Sm^SVal^SHis^+$ was the donor. Recombinants Val^R-His^+ were selected, and R_{arg}^- clones were screened as described under methods. Finally, inducibility was determined in a number of the R_{arg}^- recombinants. The results are given in Table 7. It can be seen that a multiplicity of I indexes was again obtained, ranging from 1 to 6.

TABLE 6

TRANSMISSION OF DIFFERENT I INDEXES IN THE SAME CROSS

Number of recombinants Md^+ scored	Range of Inducibility Index						
	1	1.5 to 2	2 to 3	3 to 4	4 to 5	5 to 6	6 to 7
48	2	19	6	6	8	6	1

Recipient: B Arg 5$^-$Sm^R. Donor: Hfr K10 Arg$^+$Sm^S. Recombinants: Arg$^+$Sm^R. Scoring Md: test of level of enzyme 5 in cells grown in glucose in absence of arginine. This test gives also the basal level. Test for inducibility: determination of enzyme 5 level in cells grown in glucose in the presence of 100 μg/ml of arginine. Inducibility index: ratio of enzyme 5 in cells grown in arginine over that in cells grown in minimal.

TABLE 7

THE VARIABILITY OF INDUCIBILITY INDEX AFTER CROSSING

Recipient	R^- recombinants scored	Donor: K10 $Sm^SVal^SHis^+$ Number of R^- recombinants with I index of				
		1	1.5 to 2	2 to 3	3 to 5	6
B × K10 #8 $Sm^RVal^RHis^-$	28	1	19	4	4	1

Recombinants: Val^RHis^+. Scoring I: test of behavior of enzyme 5. Inducibility index: ratio of enzyme 5 in cells grown in arginine over that in cells grown in minimal.

A second type of experiment arose from the observation that when a strain B mutant Arg 5⁻Lac⁻(B90 Lac⁻) was crossed with Lac⁺ donors, recombinants with high I indexes were frequently found. A systematic analysis was attempted on recombinants obtained from B90 Sm^RLac⁻ crossed with different donors Arg⁺-Lac⁺Sm^S. The selected recombinants were Sm^RArg⁺ and they were essentially all R⁻ (because of the use of the Sm^R character as a counterselector). They were scored for the Lac character and for inducibility. The results obtained with K10 (Table 8) suggest that some factor located in the Lac region is responsible for the appearance of I indexes higher than 2, the value found in wild type B.

These results confirm the conclusion that inducibility is controlled by several genes, one or more probably being located in the Lac region.

Discussion.—Whereas arginine represses the formation of enzymes of its biosynthetic pathway in several strains of *E. coli*, it is shown in this paper that arginine stimulated the synthesis of these enzymes in strain B and in certain B × K12 recombinants. The extent of enzyme induction varied with the strain and with growth conditions in a range from 2 to 14 times the basal activity. The ability of arginine to induce, like its ability to repress in other strains,[13] is specific for arginine: no induction was seen with other amino acids or with metabolic precursors or degradative products of arginine. Moreover, the induction is not sequential since arginine induces enzymes separated from it by a genetic block (see Table 3) just as it represses these enzymes in other strains.[13] Therefore, the induction by arginine, compared with classical induction systems, is peculiar in two ways: it is exerted by the end-product and it is not sequential.

TABLE 8

RELATION TO LAC⁺ OF HIGH INDEX OF INDUCIBILITY

		Inducibility Index in Recombinants					
		Lac⁺			Lac⁻		
Donor	% Lac⁺	Colonies analyzed	Index 2	Index 3 to 8	Colonies analyzed	Index 2	Index 3 to 8
K10	53	18	2	16	16	10	6
3000	80	33	0	33	9	1	8
P76	31	5	0	5	0	—	
		56		54	25		14
With index 3 to 8			95%			55%	

Recipient—F⁻B = Arg 5⁻ Lac⁻Sm^R (B90). Donors—Hfr K12 = Arg 5⁺Lac⁺Sm^S (K10; 3000; P76). Recombinants = Arg 5⁺ Sm^R. Inducibility index = ratio of enzyme 5 in cell grown in arginine over that in cells grown in minimal. Values of 2 is equal to that found in B. Values >2 are considered high I index.

The lack of repressibility by arginine characteristic of the B strain is obviously a requisite for the phenotypic manifestation of the inducibility by arginine. However, the two actions involve separate mechanisms, for R_{arg}⁻ mutants isolated from R_{arg}⁺ strains (W or K12) have proved to be not inducible but simply indifferent to arginine. Inducibility by arginine is a feature so far peculiar to the B strain.

This feature appears to be related to another distinctive characteristic of strain B, its low level of arginine enzymes despite the absence of repression by arginine. In contrast, the R_{arg}⁻ mutants of W or K12, which are not inducible, possess a level of these enzymes 20 to 30 times higher than that of R_{arg}⁻ strain B. When strains with a high level of enzymes were obtained from B, either by mutation or by recombination with K12, they were also found not to be inducible by arginine. It

appears, therefore, that inducibility is restricted to R_{arg}^- strains with a low enzyme level.

Since both low and high enzyme levels have been found associated with either R_{arg}^- or R_{arg}^+,[5] and at least four enzymes of the arginine sequence vary their level in parallel,[6] it was postulated that the low enzyme level in B reflected the presence in this strain of another regulatory gene, like R_{arg}, with a pleiotropic effect on the enzymes of arginine biosynthesis. The arginine pathway in *E. coli* would thus be subject to two regulatory mechanisms: end-product repression (R_{arg}) and enzyme level setting or "modulation" (Md).

The facts presented in this paper help clarify the mechanism of modulation. First of all, according to our results, the high enzyme level of Md^- mutants corresponds to structural genes working at capacity. It may be designated as the "constitutive level" because it is the level of enzyme supported by a constitutive gene, i.e., one free of both R_{arg} and Md controls. The low enzyme level of strains Md^+, in contrast, may be considered a "basal level" which can be raised by arginine. This action of arginine, which in a formal sense is that of an inducer, suggests the possibility that "modulation" might indeed involve an unknown repressor which is reversed by arginine. The function of arginine would then be that of an antirepressor, as in the usual repressor-inducer systems.

This concept is supported by the fact that in Md^+ strains the low level of enzyme is strongly dependent on the nature of the carbon source. In glucose (or lactate) the basal level is the lowest, in glycerol the highest. Since the level of the induced enzyme does not vary proportionally, the net result of shifting the carbon source from glucose to glycerol is to raise the basal level so high that the inducing effect of the arginine may be practically canceled, as in Md^- mutants. In the Md^- strains, however, the constitutive level is practically independent of the carbon source. These findings are all consistent with the hypothesis that the setting of enzyme level is due to the action of a repressor whose formation is so directly related to the general metabolism that it can be influenced by factors as nonspecific as the carbon source and temperature (see Tables 4 and 5). The situation suggests that the classical "glucose effect," described for β-galactosidase[14, 15] and for other degradative enzymes,[16] can be extended in some cases to a biosynthetic pathway.

To account for the specificity of the antagonism of arginine to repression of the arginine pathway, it seems reasonable to assume that the same repressor is formed from various carbon sources, and that different amounts of repression are due to differences in its steady-state concentration. However, a correlation between amount of repression and rate of general metabolism, as found in other systems,[16] was not found in this one. In fact, on the basis of their decreasing ability to repress, the carbon sources studied in Table 4 are arranged in the following sequence: glucose and lactate > succinate > glycerol; however, on the basis of the rate of growth they are able to support, the order would be: glucose (growth index = 1) > glycerol (growth index = 0.8) > lactate and succinate (growth index = 0.5).

By comparing the enzyme level in different strains, it is seen that Md^- is an extreme point of a range covered by the class $Md.^+$ This variety of enzyme levels is shown in Table 4 and is obtained whenever Md markers are recombined.[5] This pattern is paralleled by a similar variety of degrees of inducibility obtained in the same crosses (see Tables 6, 7, and 8). This situation formally resembles the mul-

tiplicity of phenotypic stable states of induction of β-galactosidase observed with variations in carbon source and in concentration of inducer.[15, 17] However, in the arginine system it is so far unclear whether or to what extent a genetic basis exists for this phenomenon. In any case, the behavior of Md^+ recombinant BC39 (Table 4), which is insensitive to arginine in glucose or lactate but is inducible in glycerol or succinate, underlines the reciprocal relation between Md and I. These findings suggest that the noninducibility of this strain in glucose depends on its rapid production of repressor rather than on absence of a receptor sensitive to the inducer.

The competitive nature of this inducer-repressor system is further supported by the apparent competition of endogenous arginine with the postulated non-end-product repression. Evidence for this competition was provided by the finding that the enzyme 5 level in inducible cells grown in a rich medium deprived of arginine is lower than that found in the same cells grown in minimal medium, in which the intracellular arginine concentration is presumably higher (see discussion of Table 2). Indication in the same direction was obtained when an arginine auxotroph of an inducible strain was grown at different rates in a chemostat under arginine limitation. It was found that increasing internal levels of arginine were associated with increased levels of enzyme 5. This result would appear a paradox from the point of view of end-product repression, and actually with R_{arg}^+ W or K12 strains the opposite occurs.[12, 13] However, with inducible strain BB18, in which arginine is antagonizing a "non-end-product repression," these results are what one would expect.

In conclusion, what we have called "modulation" appears to be the action of an unknown repressor derived, as in the widely known "glucose effect," via the general metabolism. Accordingly, we propose the symbol R_x, which appears to be more appropriate than Md, for this "non-end-product" type of repression, in contrast to R_{arg} for arginine repression. The nonsequential nature of induction by arginine indicates that one compound (arginine itself or a derivative) is able to reverse the R_x repression of different enzymes of its pathway. This compound may interfere either with formation of R_x or with its action. Interference with action at a single site seems unlikely at the level of the chromosome because the structural genes of arginine enzymes in *E. coli* are not clustered[18] in an operon chromosomal unit.[19] Remaining possibilities include interference with the formation of R_x or with its action at multiple sites or at a single ribosomal cluster of sites making different arginine enzymes. A discrimination among these alternatives is not possible with the data presented here concerning enzymes 5 and 7 only. For, given the lack of gene clustering in the arginine pathway, the parallel behavior of enzymes 5 and 7 cannot be generalized to the other enzymes of this sequence until they are individually tested.

Summary.—Whereas arginine represses the formation of enzymes of the arginine biosynthetic pathway in several strains of *E. coli*, it is shown to stimulate the synthesis of certain of these enzymes in strain B. This strain has a low "basal" enzyme level controlled by "modulation"; mutants of strain B that have high levels of the enzymes are not further stimulated by arginine. In strains with low or moderate basal levels, a shift in carbon source can influence the formation of these biosynthetic enzymes in a manner similar to the "glucose effect" observed with

many inducible degradative enzymes.

These results suggest that modulation involves repression by a "non-end-product" formed via general metabolism, and induction by arginine involves reversal of this repression. The symbol R_x is proposed, instead of Md, for this non-end-product repression as opposed to R_{arg} for the end-product repression.

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† The schematic outline of arginine biosynthesis is given below. The numbers refer to the enzymes that catalyze each reaction.

$$\text{glutamate} \xrightarrow{1} \text{acetylglutamate} \xrightarrow{2} \text{acetylglutamatesemialdehyde} \xrightarrow{3}$$
$$\text{acetylornithine} \xrightarrow{4} \text{ornithine} \xrightarrow{5} \text{citrulline} \xrightarrow{6} \text{arginine-succinate} \xrightarrow{7} \text{arginine}$$

‡ The following abbreviations are used for the genetic markers: Arg = arginine; His = histidine; Lac = lactose; Sm = streptomycin; Val = valine; R_{arg} = repressibility by arginine; Md = modulation or enzyme level setting; I = inducibility; R_x = non-end-product repressibility.

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THE NATURE OF INTERGENERIC EPISOMAL INFECTION

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Evidence has now been accumulated to bear out the hypothesis that interspecies genetic exchange will occur only among organisms which have similar deoxyribonucleic acid (DNA) base compositions. This has been found to be true among the T-even coliform bacteriophages,[1a,b] the *Brucellaceae*,[2] the *Bacillaceae*,[3] the *Neisseriaceae*,[4] and the *Enterobacteriaceae*,[5] the former three bacterial groups by means of transformation and the latter by means of transduction and conjugation. Recently, a different means of nonintegrated genetic exchange mediated by episomal infection[6, 7] has been shown to take place among the members of the *Enterobacteriaceae* which differ significantly in their DNA base compositions.[8a,b, 9]

The genetic determinants of bacteria normally comprise an integral part of the structure of the chromosome and are always present in one or another of their allelic forms. There is a class of genetic elements, however, which can be either present or absent and which, in the cell, can replicate synchronously or autonomously with respect to the bacterial genome. For these added elements, Jacob and Wollman have proposed the name *episomes*.[6]

The process of conjugation in the *Enterobacteriaceae* (which has been observed only among organisms of similar DNA base composition) involves a sexual differentiation which has been ascribed to the presence of a fertility factor in the donor strain (or male) and its absence in the recipient strain (or female). The fertility factor possesses remarkable properties in that it may exist either in an integrated state, in which it replicates as part of the bacterial genome (Hfr-male), or in an autonomous state, in which it behaves as if it were independent of the bacterial genome (F^+-male);[10, 11] hence, the fertility factor belongs to the episomic class of genetic elements. Although it appears that the integrated and nonintegrated states are mutually exclusive, unstable Hfr strains have been described in which both states appear to be in equilibrium[12] and in these variant strains the fertility factor is always attached at the same site of the bacterial chromosome when in the integrated state.[13] Two variants of this type have been isolated in which the fertility factor has incorporated a part of the bacterial chromosome one (F-Lac) which carries a determinant of β-galactosidase synthesis and the other (F-Pro) which carries a determinant of proline synthesis,[11] and is able to transfer these chromosomal segments to suitable F^- recipient strains. In this process of genetic transfer which has been termed F-duction, the fertility factor and the particular chromosomal segment are transferred exclusively and hence the genetic transfer is analogous to the process of transduction in which a temperate bacteriophage may transfer a part of the host genome to a recipient bacterium.[14, 15]

Escherichia coli and *Salmonella* (50% guanine + cytosine) will transfer several episomal elements to *Serratia marcescens* (58% guanine + cytosine).[8a, b] Since the buoyant density of DNA in CsCl has been found to be a function of its G-C content,[16, 17] the banding positions of the DNA of both parent strains will be different

in a CsCl density gradient; moreover, their band profiles indicate that they possess essentially no molecules of similar base composition in common. Thus the technique of density gradient centrifugation[18] provides a unique method of detecting genetic transfer between *E. coli* or *Salmonella* and *Serratia*. Furthermore, fractionation by preparative density gradient centrifugation of the DNA isolated from the episomally infected recipient makes possible the detection of relatively small amounts of DNA which would not be detectable in the unfractionated sample.

Genetic Evidence for Episomal Transfer.—A strain of *Salmonella typhosa* carrying the fertility factor, F, of *Escherichia* which harbors a segment of the bacterial chromosome governing lactose utilization (F-Lac) was used in this study. This organism is capable of transmitting donor ability and lactose utilization exclusively and with a relatively high efficiency to various species and strains of *Escherichia*, *Vibrio*, *Salmonella*, *Shigella*, and *Serratia*. The transfer of this episomal element has been found to be independent of any other genetic marker and the kinetics of transfer are similar to those found for the transfer of the sex factor in $F^+ \times F^-$ matings. It has been found that the episomal element of the recombinant culture replicates as a single unit independent of the host genome or at best undergoes a rapid alternation between the integrated and nonintegrated states.[7, 8a,b, 11] A more detailed description of this case of intergeneric episomal transfer has been reported in a separate communication.[8a,b]

The Detection of Donor DNA in the Recipient Organism.—The episomally infected *S. marcescens* strain was cultured from a single clone and the DNA isolated by the method of Marmur.[19] In addition, DNA was isolated from the *S. marcescens* parent strain and from an F^+ strain of *E. coli* by the same procedure. The DNA was banded in a CsCl density gradient in the Spinco Model E Analytical Ultracentrifuge at 44,770 rpm. When equilibrium was reached, photographs were taken using ultraviolet absorption optics. Figure 1 shows microdensitometer tracings of the resultant band profiles of the DNA isolated from the *S. marcescens* parent strain (A), the episomally infected *S. marcescens* (F-Lac) (B), and, to illustrate the correspondency in density of the new band appearing in (B) to that of the donor strain, a final tracing shows the band profile of the DNA isolated from an F^+ strain of *E. coli* (C). The band of heaviest density is that of a reference standard (N^{15}-labeled *Pseudomonas aeruginosa* DNA[20]) which is used to calculate the density of the other bands. It can clearly be seen that the unfractionated episomally infected *S. marcescens* DNA gives rise to two distinct bands in the CsCl density gradient, whereas the unfractionated *S. marcescens* parent strain does not. Furthermore, the buoyant density of the new band appearing in (B) corresponds to the buoyant density of the DNA isolated from the F^+ donor strain of *E. coli*. The buoyant density of the heavier band (1.718 gm/cm^3) corresponds to a G-C content of 58% (equivalent to that of the *S. marcescens* parent strain), whereas the lighter band (1.709 gm/cm^3) corresponds to a 50% G-C content (equivalent to that of the *E. coli* F^+ donor strain). The lighter band represents approximately 1% of the total DNA of the recombinant. When the DNA from the recombinant is thermally denatured and quickly cooled, the buoyant densities of both bands increase 0.015 gm/cm^3 in agreement with previous observations,[16] whereas the DNA isolated from the parent *S. marcescens* gave rise to a single band with a corresponding density increment under the same conditions.

The DNA isolated from the episomally infected *S. marcescens* was also studied after it had been fractionated using CsCl preparative density gradient centrifugation. This procedure was deemed advantageous in that it would provide a more sensitive method for the detection of DNA transferred from the donor strain to the recipient and it would also make possible a closer study of the transferred DNA by the removal of a large proportion of the normal DNA complement of the recipient *S. marcescens*. In a typical experiment approximately 300 μg of the DNA isolated from the recombinant was dissolved in a concentrated solution of CsCl and the density adjusted to approximately 1.710 gm/cm^3; this solution was then centrifuged in a lusteroid tube in the Spinco Model L Preparative Ultracentrifuge using the SW39 swinging bucket rotor. Normally the material was centrifuged at 35,000 rpm for approximately 24 hours and then the speed reduced to 20,000 rpm and the run continued at this speed for 4–5 days. At the end of this period the rotor was allowed to coast (unbraked) to a stop and the solution fractionated in an apparatus modeled after that of Szybalski[21] by puncturing a hole in the bottom of the lusteroid tube and collecting drops. After appropriate dilutions, absorbances at 260 mμ were measured on alternate fractions using a Beckman DU Spectrophotometer. The curve obtained by plotting absorbance versus fraction number is shown in Figure 2. Although no separate bands corresponding to the transferred DNA appeared in this plot, by centrifugation in the analytical ultracentrifuge of the indicated fractions (Fig. 2) selected from the less dense side of the band profile, the DNA introduced into the recipient *S. marcescens* could be readily resolved from its normal DNA complement. The results of this procedure are shown in Figure 3; in addition to confirming the presence of the band of buoyant density 1.709 gm/cm^3 as observed in the analytical ultracentrifuge on the unfractionated sample (Fig. 1B), the presence of a second band of buoyant

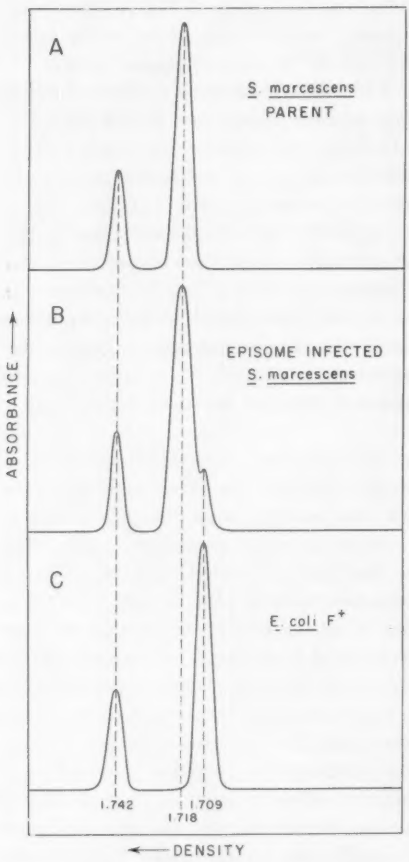

FIG. 1.—Band profiles of unfractionated DNA from parental and recombinant strains. Microdensitometer tracings of ultraviolet absorption photographs of samples at equilibrium in a CsCl density gradient at 44,770 rpm. The ordinate represents DNA concentration as a function of the distance from the axis of rotation. The band of buoyant density 1.742 gm/cm^3 (N^{15}-labelled *Pseudomonas aeruginosa* DNA) is used as a reference standard. See text for a description of samples.

density 1.703 gm/cm³ (44% G-C) was observed. This band is not detectable in the analytical ultracentrifuge run on the unfractionated DNA of the recombinant since it is present only to the extent of 0.1–0.2% of the total DNA sample.

In a control experiment the DNA isolated from the *S. marcescens* parent strain was fractionated and subsequently banded in the analytical centrifuge according to the above procedure. Although a band of buoyant density of approximately 1.709 gm/cm³ was observed in this case, it comprises less than 0.1% of the total DNA sample; similar bands have been observed in another preparation of *S.*

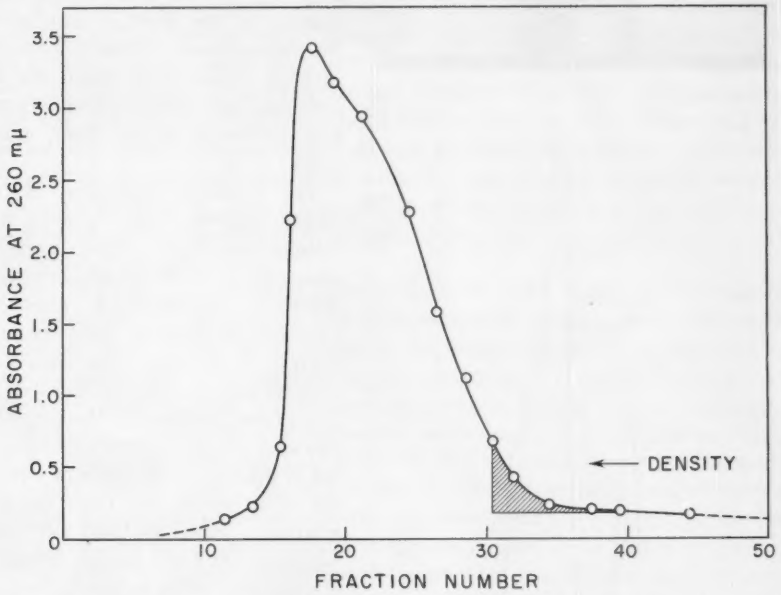

FIG. 2.—Preparative density gradient fractionation of DNA from *Serratia-coli* recombinant. Absorbances were measured at 260 mμ on a Beckman DU Spectrophotometer after appropriate dilution. Fractions were 0.06 ml each; above absorbances represent dilution of 1:2.67. Skewness of the band is an artifact resulting from the large amount of DNA which had to be banded in order to obtain sufficient amounts of the transferred DNA. For a description of the fractionation procedure, see text. Shaded area represents fractions subsequently centrifuged in analytical centrifuge (Fig. 3).

marcescens DNA, as well as in several preparations of DNA from *Aerobacter aerogenes*.[22] In none of these preparations, however, was a band of buoyant density 1.703 gm/cm³ observed.

Discussion.—The nature of the vectors involved in the transfer of genetic information among bacterial species has been elucidated in the process of transformation,[23] transduction,[14] and conjugation.[24] In all of these processes of genetic recombination DNA is implicated as the underlying principle of genetic specificity of the transferred material. The process of episomal transfer has not been so extensively studied as a means of genetic exchange, particularly in regard to the nature of the episomal element. Genetic analysis has shown that the fertility factor is able to ensure the transfer of small chromosomal segments by a process

which is similar in many respects to that of specialized transduction. In addition, the fertility factor and the F-duced chromosomal segment are transferred exclusively and the episomal element may not be integrated into the genome of the recipient bacterium since the recombinants carry both parental alleles.[7, 11] In a recent communication,[25] Lavalle and Jacob have reported that several episomal elements are sensitive to the disintegration of P^{32} in the same manner as the genetic material of the chromosome. From the results of their study it is reasonable to conclude that episomal elements are high molecular weight DNA molecules.

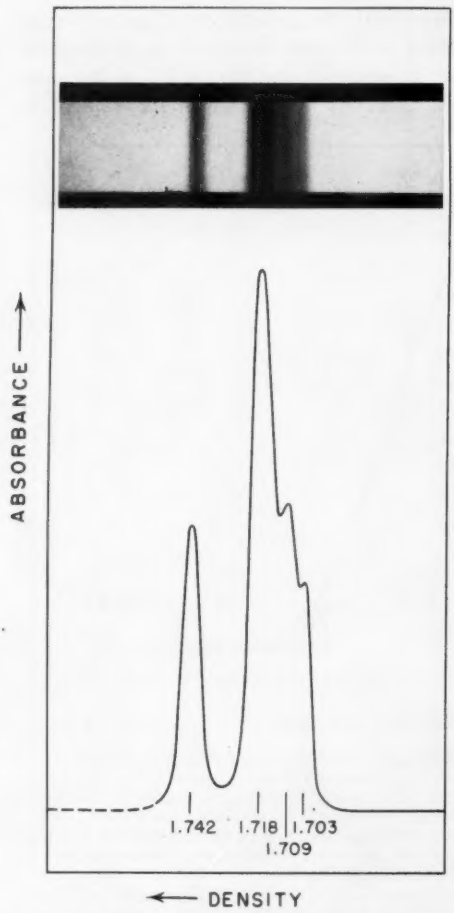

FIG. 3.—Band profile of fractionated DNA of *Serratia-coli* recombinant. Analytical ultracentrifuge run on fractionated *Serratia-coli* recombinant DNA. Fractions centrifuged are indicated in Fig. 2. Ultraviolet absorption photograph taken after equilibrium was reached at 44,770 rpm (top). Microdensitometer tracing of ultraviolet absorption photograph (bottom). The band of buoyant density 1.742 is used as a reference standard (N^{15}-labeled *Pseudomonas aeruginosa* DNA).

The evidence presented above clearly indicates that genetic exchange can occur among bacteria whose DNA base compositions are different. Furthermore, although cytoplasmic factors may be involved, it is clear that episomal transfer does involve a transfer of DNA from the donor to the recipient cell since separate bands corresponding in density to both parent strains are observed in the CsCl density gradient. Since the band width obtained in the CsCl density gradient is a function of the molecular weight of the banded DNA,[18] it is also evident that the transferred DNA is of a molecular weight comparable to that of the donor and recipient strains, since similar band profiles are obtained for the banded DNA of both parent strains and the transferred DNA. It is also reasonable to conclude that two types of high molecular weight DNA are transferred since two separate bands of different G-C contents are observed after the DNA isolated from the episomally infected recipient has been fractionated using CsCl preparative density gradient centrifugation. The results presented in this study are certainly consistent with the hypothesis that the genetic component of the episomal element is deoxyribonucleic acid.

No attempt will be made here to identify the two observed bands in terms of their genetic function; this is the object of a further study. It is striking, however, that the physical-chemical evidence is consistent with the genetic evidence in indicating the involvement of two distinct characters in the process of episomal transfer. In the absence of any positive identification of the functional nature of the two observed minor bands, any interpretation of this observation must remain purely a matter of speculation.

It is interesting that no bands of buoyant density intermediate between those of the donor and recipient parent strains are observed in the CsCl density gradient. If it is assumed that a genetic recombination between the episomal element and the *S. marcescens* host genome would result in a *hybrid* DNA of an average G-C content intermediate between those of the donor and recipient parent strains, then this *hybrid* should band between the two parent strains. Since no intermediate band was observed, even after the DNA isolated from the recombinant had been fractionated using CsCl preparative density gradient centrifugation, the evidence presented in this paper is consistent with the view that the episomal element is not integrated into the host genome by a process of genetic recombination, but rather replicates independently in the cytoplasm or in some unknown association with the host genome.

DNA isolated from bacterial sources shows a small degree of heterogeneity with respect to base composition and a few successful attempts have been made to fractionate nucleic acids with respect to differences in biological and physical-chemical properties. The interspecific transfer of genetic material among organisms which differ in base composition represents a unique method of *biological* fractionation. Since several chromosomal segments have already been F-duced and it seems likely that any gene of the bacterial chromosome may become incorporated into a fertility factor and, hence, exhibit the properties of episomal elements, intergeneric episomal transfer and subsequent fractionation in the CsCl density gradient may be used to isolate various regions of the donor genome.

The problem of contamination must be considered in a discussion of the results presented in this paper since the two bands observed after fractionation of the DNA isolated from the recombinant represent a relatively small fraction of its total DNA complement. For this reason the episomally infected *S. marcescens* was cultured from a single clone as described above. The buoyant densities of the episomal bands, as well as their relative proportions, were reproducible in DNA preparations from recombinants obtained from several different crosses. If contamination is to be assumed a factor in these results, then it must be postulated that a *double* contamination by two bacterial species whose respective G-C contents are 50 per cent and 44 per cent has occurred; moreover, this double contamination must occur in the same proportions. We feel that this is an improbable chain of events.

The possibility that other factors may be responsible for observed decrease in buoyant density of a small fraction of the DNA isolated from the recombinant must also be considered. For example, could protein-DNA aggregates account for the small fraction of DNA of lighter buoyant density observed in the CsCl density gradient? Extensive studies on the DNA isolated from *E. coli* K-12 under a wide range of conditions have always shown it to have the same buoyant

density in CsCl.[26] All unfractionated bacterial DNA preparations which have been banded in CsCl have been observed to have unimodal DNA distributions.[16,17,27,28] The DNA obtained from direct lysates of numerous bacterial preparations have always been observed to form unimodal, sharp bands of the same buoyant density as extensively deproteinized preparations.[29] Finally, all DNA preparations which have been examined to date, irrespective of the source of the DNA and the method used for its isolation, have been observed to fit the linear relationship established between buoyant density in CsCl and G-C content,[28] with the exception of the DNA isolated from the T-even coliform bacteriophages which contain an unusual base (5-hydroxymethylcytosine) which may be glucosylated to varying degrees.

Summary.—Genetic exchange can occur among organisms which differ in DNA base composition. The results presented in this study are consistent with the genetic evidence that episomal elements are deoxyribonucleic acid and that the transferred material is not integrated into the genome of the recipient bacterium. It has also been shown that the transfer of the (F-lac) episome involves two distinct species of DNA whose base compositions are 50 per cent and 44 per cent G-C respectively.

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*BIOPHYSICAL STUDIES OF BROAD BEAN MOTTLE VIRUS**

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Viruses are useful test objects for studying structural and functional relationships between proteins and nucleic acids. For these purposes it is desirable, at least for the present, to deal with viruses that are as small as possible with the expectation that small size and structural simplicity will go hand in hand. The members of the group of so-called *spherical viruses* perhaps approach this desideratum most closely. Among the most extensively studied are the plant viruses turnip yellow mosaic virus,[1] tomato bushy stunt virus,[2] wild cucumber mosaic virus,[3] southern bean mosaic virus,[4] and squash mosaic virus,[5] the bacterial virus ϕX174,[6] and polio virus.[7] All have molecular weights in the range 5–10 $\times$ 10^6 and nucleic acid content in the range 1.5–2.5 $\times$ 10^6 (in molecular weight units). Recently it has been shown that the molecular weight of bromegrass mosaic virus[8] is even lower —4.6 $\times$ 10^6—and that its content of nucleic acid is only 1 $\times$ 10^6.

The data to follow show that broad bean mottle virus (BBMV) is also quite low in molecular weight—5.2 $\times$ 10^6—and in nucleic acid content—1.1 $\times$ 10^6. This virus could become a useful complement to bromegrass mosaic virus since the two differ markedly in both amino acid and nucleotide composition. Indeed, because its yield from infected tissues is exceedingly high, broad bean mottle virus could be the more favorable subject for structural studies.

Broad bean mottle virus was first isolated and purified by Bawden, Chaudhuri, and Kassanis[9] who investigated some of its biological properties. Serological and some chemical properties have been studied by Wetter, Paul, Brandes, and Quantz.[10] Wittmann and Paul[11] determined its amino acid composition.

Methods.—Isolation and purification procedures: The virus was obtained from a stock culture kept by F. C. Bawden.[12] It was inoculated onto horse bean (*Vicia faba* L. var. broad windsor) and was isolated from infected leaves 3 weeks later.

Infected leaves, frozen at −25°C., were homogenized in a Waring blendor in cold potassium phosphate buffer (0.01 *M* in phosphate, pH 7). In some isolations as much as 0.01 *M* ascorbic acid was added to this solution in order to prevent oxidation of cellular constituents. An equal volume of a 1:1 n-butanol-chloroform mixture was added to the homogenate and the resulting mixture was shaken gently for 15 min. The emulsion was broken by a low speed centrifugation. The aqueous layer was removed and was immediately centrifuged in a No. 30 rotor in a Spinco Model L preparative centrifuge at 15,000 rpm for 15 min. The supernatant was then centrifuged at

28,000 rpm for 150 min. The pellets were resuspended in phosphate buffer. The resulting solution was clarified by low speed centrifugation and then centrifuged in the No. 40 rotor at 38,000 rpm for 90 min. After another similar cycle of differential centrifugation the colorless pellets were resuspended in phosphate buffer at 4° (0.1 *M* in phosphate, pH 7). After a final low speed centrifugation, the material was used immediately or frozen at −25°. The yield is exceedingly high—150 mg per 100 gm tissue.

It will be shown in the next section that the above procedure results in a single macromolecular component. Analyses of zonal centrifugation of the preparations in sucrose density gradients in the manner described by Bockstahler and Kaesberg[8] indicated that infectivity and this component sedimented at the same rate.

Since the virus has some physical similarities to bromegrass mosaic virus, reciprocal serological tests were carried out.[13] The results showed that there was no antigenic relationship.

Analytical procedures: Analytical sedimentation studies were made in the 12 mm cell of a Spinco Model E analytical ultracentrifuge. All runs were made at approximately 20°. The sedimentation coefficients were corrected to conditions obtaining in water at 20°. The buffer was 0.10 ionic strength, pH 7.0 (0.0067 *M* NaH_2PO_4, 0.0133 *M* Na_2HPO_4, 0.0534 *M* NaCl).

Electrophoretic studies were made in a Spinco model H electrophoresis-diffusion apparatus. Sodium acetate and phosphate buffers were used at ionic strengths 0.10.

Diffusion measurements were made in the same apparatus. A sharp boundary was created between the solvent and the virus solution in a standard Tiselius cell and the change in concentration of the solute as a function of distance and of time was followed by means of the Rayleigh interference optical system. The diffusion was allowed to proceed at 2° for a period of 3 days. The buffer was the same as that used for sedimentation experiments. Apparent diffusion coefficients were calculated from the fringe data according to the method of Longworth.[14] The diffusion coefficient, corresponding to infinite time, was obtained from a plot of the apparent diffusion coefficients against the reciprocal of time. This value was corrected to conditions in water at 20° under the assumption that the diffusion rate was linearly dependent upon viscosity and absolute temperature.

The partial specific volume of the virus was obtained from measurements of concentration and of densities of the solution and solvent. Densities of the virus solutions were measured by pycnometry at 25°. The concentration of the solution was obtained by the difference in dry weights of known volumes of solvent and solution.

Ultraviolet absorption measurements were obtained with a Cary Model 11 spectrophotometer, in the same buffer as that used for the sedimentation experiments. The absorption was corrected for the optical density due to light scattering.

For the determination of nucleotide composition, the lyophilized virus, without prior removal of protein, was digested in 0.5 *N* potassium hydroxide at 37° for 20 hours with shaking. The separation of nucleotides from the protein and the chromatographic separation of the four nucleotides were made according to the procedure of Osawa and associates.[15] Chromatography was carried out on Dowex 1 (×2), 200–400 mesh. Continuous elution was used with the formic acid system originally developed by Hurlbert and associates.[16] The phosphorus content of the nucleic acid was calculated from the nucleotide composition.

Phosphorus content of the virus was determined in triplicate by the method of Allen[17] on 3 separately purified samples. The nucleic acid content of the virus was calculated from the phosphorus content in the virus and in the nucleic acid. The nucleic acid content was also determined by means of the optical method of Englander and Epstein.[18] For this procedure the absorbancy index of the nucleic acid *as it exists in the virus* was calculated from the nucleotide composition, the absorbancy indices of the constituent nucleotides,[19] and an assumed hypochromicity of 1.50 for the nucleic acid.[20] The absorbancy index for protein was taken to be $^1/_{40}$th that of RNA. The nucleoprotein concentration was determined refractometrically. The specific refractive increment was taken to be 18×10^{-5} for a 1 mg per ml solution.

Results.—Sedimentation: A single sharp peak is seen in the analytical ultracentrifuge as shown in Figure 1. The sedimentation coefficients have a concentration dependence (Fig. 2) approximately expressed by

$$s_{20,w} = (84.8 - 0.47c)S$$

where c is concentration in mg per ml.

Electrophoresis: When the virus is examined electrophoretically at pH 7.0, only one peak is resolved (Fig. 3) even in runs lasting more than 5 hours. At this pH the virus is negatively charged and the mobility is -6.9×10^{-5} cm^2 per volt per second. Unpublished studies by J. S. Semancik and J. B. Bancroft (personal communication) show that the virus migrates as a single peak over the entire pH range 3.0–8.0.

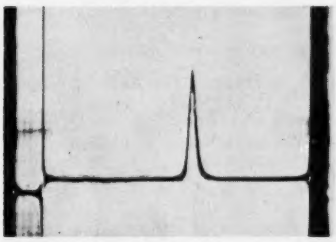

FIG. 1.—Sedimentation pattern of broad bean mottle virus at a concentration of 3.55 mg/ml. Photographed 14 min after the rotor reached speed of 35,600 rpm. Direction of sedimentation is to the right.

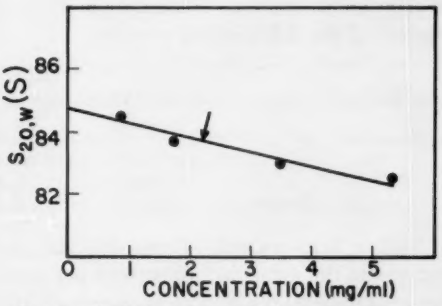

FIG. 2.—Sedimentation coefficients plotted against concentration of the virus. The arrow shows the concentration at which the diffusion experiment was made.

Diffusion: The diffusion coefficient at 2° in the buffer used is 0.766×10^{-7} cm^2 per second. The diffusion coefficient, $D_{20,w}$, corresponding to a temperature of 20° in a solvent with the viscosity of water is 1.380×10^{-7} cm^2 per second.

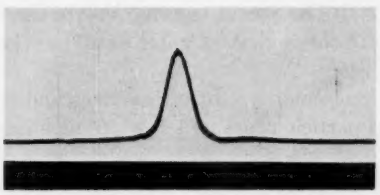

FIG. 3.—Electrophoretic pattern (descending pattern) of broad bean mottle virus in 0.1 ionic strength phosphate buffer (pH 7.0) after 322 min at 1.93 volt/cm.

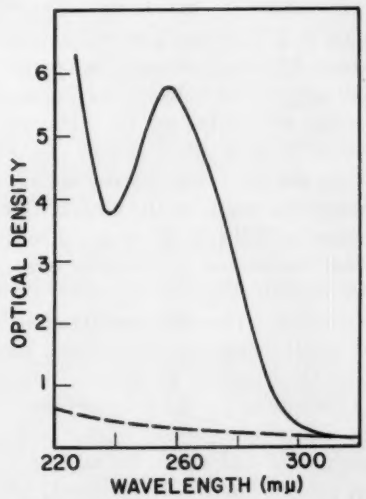

FIG. 4.—Optical density plotted against wavelength for broad bean mottle virus at a concentration of 1 mg/ml.

Partial specific volume: The partial specific volume of the virus is 0.717 ml per gm.

Ultraviolet absorption: Figure 4 shows the ultraviolet absorption curve for a 1 mg per ml virus solution at pH 7.0. The ratio of the absorbancy at 260 mμ to that at 280 mμ, after correction for light scattering, is 2.1. The curve has a maximum at 258 mμ and a minimum at 238 mμ. The absorbancy index at 260 mμ is 5.40 cm^2 per mg.

Nucleotide composition: The molar nucleotide residue composition of the RNA of the virus is given in Table 1. From the nucleotide composition the phosphorus content of the RNA is 9.62 per cent.

TABLE 1

THE MOLAR NUCLEOTIDE RESIDUE COMPOSITION IN THE RNA OF BROAD BEAN MOTTLE VIRUS

Preparation	Cytidylic acid	Adenylic acid	Guanylic acid	Uridylic acid
I	19.9	27.2	25.1	27.8
II	18.9	27.4	24.1	29.6
Average	19.4	27.3	24.6	28.7

Nucleic acid content: From the phosphorus determinations, the phosphorus content of the virus is 2.12 ± 0.10 per cent. Since the nucleic acid contains 9.62 per cent phosphorus, the virus contains 22.0 ± 1.0 per cent RNA.

The absorbancy index of the RNA, calculated from the nucleotide composition and an assumed hypochromicity, is 23.4 cm^2 per mg. The absorbancy index for the virus was found (above) to be 5.40 cm^2 per mg. Thus from Englander and Epstein's equation[18] the virus contains 21.1 ± 1.0 per cent RNA.

Molecular weight of the virus: Molecular weight is calculable from Svedberg's equation

$$M = \frac{sRT}{D(1 - \bar{v}\rho)}$$

where s, R, T, D, $\bar{v}$, and ρ are sedimentation coefficient, gas constant, absolute temperature, diffusion coefficient, and partial specific volume, and density of the solvent respectively. The diffusion coefficient $D_{20,w}$ is 1.38×10^{-7} cm^2 per sec at a concentration of 2.22 mg per ml. The sedimentation coefficient $s_{20,w}$ at this concentration is 83.8×10^{-13} second (see Fig. 2). The partial specific volume, $\bar{v}$, is 0.717 ml per gm. The solvent density is, of course, that of water at 20°. Thus the molecular weight of the virus is 5.20×10^6.

Amount of RNA in the virus: From the molecular weight of the virus and the per cent nucleic acid in the virus, each virus particle contains 1.1×10^6 molecular weight units of RNA.

Discussion.—The small amount of nucleic acid contained in viruses such as bromegrass mosaic virus and broad bean mottle virus is of special significance. Ultimately, the complete structure of nucleic acids (or at least their base sequences) will be required for the interpretation of genetic data in chemical terms. For a given virus, the amount of nucleic acid it contains is, presumably, a measure of the magnitude of this task. By way of comparison, tobacco mosaic virus, which is being studied intensively in several laboratories, contains about 6,000 nucleotide residues per particle, bromegrass mosaic virus contains about 3,000, and broad bean mottle virus contains about 3,400.

The sizes of the protein subunits are not known for bromegrass mosaic and broad bean mottle virus, although preliminary experiments in both cases suggest that they will consist of roughly 190 amino acid residues. Thus the coding ratio would be about 17.

Summary.—Broad bean mottle virus has been obtained as an electrophoretically and ultracentrifugally homogeneous preparation. The molecular weight of the virus was found to be 5.20×10^6, based on a sedimentation coefficient of $(84.8-0.47c)S$ and a diffusion coefficient of 1.38×10^{-7} cm^2 per second and a partial specific volume of 0.717 ml per gm. The virus contains about 1.1×10^6 molecular weight units of ribonucleic acid.

* This investigation was supported by the U. S. Public Health Service and by the National Science Foundation.

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AN EFFECT OF L-LEUCINE AND OTHER ESSENTIAL AMINO ACIDS ON THE STRUCTURE AND ACTIVITY OF GLUTAMIC DEHYDROGENASE

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Glutamic dehydrogenase (GDH) catalyzes the interconversion of α-ketoglutarate (α-KG), a Krebs cycle component, and L-glutamate. With the crystalline protein from beef liver, it has been shown that the activity of the enzyme, which is composed of subunits, depends on its state of aggregation.[1] Our recent finding that several steroid hormones promote the reversible dissociation of the enzyme into subunits[2] suggested that control of the physical state of this protein, and perhaps of others, may be a means by which enzymic reactions are regulated. This idea

seems especially attractive since we have also found[3] that the subunits are enzymically active, but have a different substrate specificity from that of the aggregate.

Experiments described in the present communication suggest that essential amino acids could also regulate glutamate metabolism, since certain essential amino acids can affect the structure (and function) of GDH.

Materials and Methods.—Steroid hormones, diethylstilbestrol, nucleotides, and a suspension of crystalline beef liver glutamic dehydrogenase in Na_2SO_4 were obtained from the Sigma Chemical Company. The amino acids were purchased from Nutritional Biochemical Corporation, and phenanthridine was produced by Aldrich Chemical Company, Inc.

Mitochondria, separated from rat liver according to the technique of Hogeboom,[4] were suspended in 0.025 *M* phosphate buffer pH 7.5, containing 0.75 *M* KCl. These suspensions, 1 ml of which was equivalent to 125 mg of the original tissue, were frozen and thawed four times before use.

Glutamic dehydrogenase activity was assayed spectrophotometrically by following oxidation or reduction of the pyridine nucleotide.[2] Sedimentation experiments were performed in the Spinco model E analytical ultracentrifuge as a rotor speed of 59,780 essentially as described earlier.[2]

Results.—Glutamate oxidation by either DPN or TPN, catalyzed by crystalline glutamic dehydrogenase prepared from beef liver, was stimulated by L-leucine (Fig. 1, curves A and B). During the course of these experiments, four different

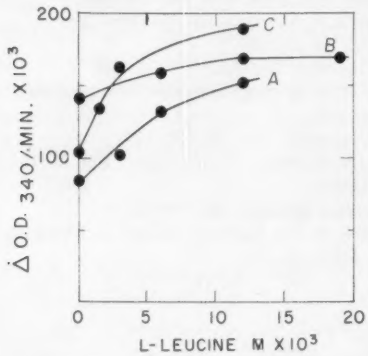

FIG. 1.—Effects of L-leucine on glutamate oxidation. *A*, *B*: 2.5 ml reaction mixture contained 0.01 *M* tris buffer pH 8.0, EDTA 1×10^{-4} *M*, glutamate 0.1 *M*, DPN 2×10^{-4} *M*, 0.01 mg of crystalline beef liver glutamic dehydrogenase (see text) and L-leucine as shown; *B*: 2.5 ml. reaction mixture contained 0.01 *M* tris buffer pH 8.0, EDTA 1×10^{-4} *M*, α-ketoglutarate 5×10^{-3} *M*, NH_4Cl 0.1 *M*, DPNH 5×10^{-5} *M*, mitochondrial suspension equivalent to 1.25 mg of rat liver, and L-leucine as shown.

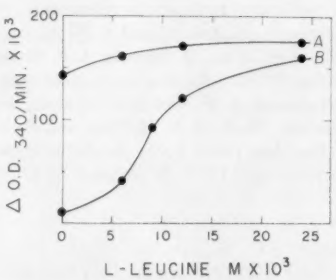

FIG. 2.—Effect of L-leucine in inhibition of glutamic dehydrogenase by diethylstilbestrol. Experiment as in Figure 1 *A*, *B* except for presence of ethanol 0.4% in each, and diethylstilbestrol, 8×10^{-6} *M* in *B*.

preparations of the crystalline enzyme were examined, and the extent of stimulation by 1.2×10^{-2} *M* L-leucine varied from 20 to 100 per cent depending on the preparation. This monocarboxylic amino acid also stimulated GDH activity in mitochondrial preparations from rat liver (Fig. 1, curve C). In the latter case 1.5×10^{-3} *M* L-leucine caused a 30 per cent stimulation of glutamate oxidation, and 1.2

$\times 10^{-2}$ *M* doubled the rate. As expected, no oxidation of L-leucine could be observed with the amount of enzyme used in the present experiments. This is consistent with the observation of Struck and Sizer[5] that although GDH catalyzes the oxidation of L-leucine, this occurs at a rate only 1.7 per cent that of L-glutamate. It should be emphasized that leucine stimulates the oxidation of glutamate rather than vice versa. This is attested to by the finding that leucine stimulates equally well the reduction of α-KG.

D-leucine had virtually no effect on the GDH reaction and, moreover, when used in equimolar concentrations with the L-isomer, did not reduce the stimulation produced by the latter. Neither the α-keto analog of leucine, α-ketoisocaproate, nor isocaproate itself influenced the rate of the enzymic reaction. Leucinamide, leucylleucine, and leucylglycine were also ineffective. Methionine, isoleucine, and the unnatural amino acid norvaline, though considerably less active, were also able to stimulate the GDH reaction. Further effects of these amino acids will be discussed below. Glycine, alanine, valine, norleucine, lysine, arginine, ornithine, serine, threonine, phenylalanine, tyrosine, tryptophan, α-aminobutyrate and histidine had no influence on the GDH reaction.

Mechanism of amino acid stimulation of GDH: Diethylstilbestrol, a steroid analog, inhibits the GDH reaction by dissociating the enzyme molecule into subunits.[2] As shown in Figure 2, 2.5×10^{-2} *M* L-leucine completely overcame the 91 per cent inhibition of the crystalline enzyme resulting from 8×10^{-6} *M* diethylstilbestrol. Again L-methionine, L-isoleucine, and L-norvaline could overcome the steroid inhibition but were somewhat less effective than L-leucine. Table 1 shows the concentrations of these compounds required to restore 50 per cent of the enzyme activity after inhibition by 8×10^{-6} *M* DES.

TABLE 1

ABILITY OF AMINO ACIDS TO RESTORE GDH ACTIVITY AFTER INHIBITION WITH DIETHYLSTILBESTROL

Amino acid	Conc. to restore 50% of GDH activity after inhibition with 8×10^{-6} *M* diethylstilbestrol
L-leucine	8×10^{-3} *M*
L-methionine	1.6×10^{-2} *M*
L-isoleucine	3.6×10^{-2} *M*
DL-norvaline	6×10^{-2} *M*

Experiment conducted as in Figure 2 except for additions as noted.

Phenanthridine, like DES,[2] inhibits the GDH reaction by disrupting the enzyme molecule.[6] L-leucine was also able to overcome the inhibition produced by this compound (Table 2).

Frieden[1] has made the observation that relatively high concentrations of DPNH can disaggregate the GDH molecule and thus inhibit the enzymic reaction. The inhibitory effect of the reduced pyridine nucleotide could also be prevented by L-leucine. Curve A in Figure 3 shows the inhibition of α-ketoglutarate reduction at higher concentrations of DPNH. As shown by curve B, the presence of 1.2×10^{-2} *M* L-leucine prevented this effect of DPNH.

Since L-leucine could antagonize the inhibitory action of a number of compounds known to dissociate the GDH molecule into subunits, it seemed likely that this

amino acid could prevent such reagents from dissociating the enzyme. Accordingly, the effects of L-leucine on the DES-induced disruption of the enzyme were studied in the ultracentrifuge. Figure 4 (curve A) illustrates the alteration of GDH in

TABLE 2

EFFECT OF PHENANTHRIDINE AND L-LEUCINE ON GDH ACTIVITY

Additive	$\Delta O.D._{340}/min \times 10^{-3}$
None	156
Phenanthridine $2 \times 10^{-4} M$	10
Phenanthridine $2 \times 10^{-4} M$ + L-leucine $1.2 \times 10^{-2} M$	180

Experiment performed as in Figure 1A, B except for presence of $2 \times 10^{-4} M$ phenanthridine and 1% ethanol.

the presence of $2 \times 10^{-4} M$ DES, as shown earlier.[2] Curve B shows the same experiment with the addition of $2.4 \times 10^{-2} M$ L-leucine. It is apparent that the amino acid can, indeed, suppress the disaggregation of the GDH molecule caused by DES.

It appears from these experiments that L-leucine has an effect on the enzyme very similar to that reported for DPN and ADP.[7] These nucleotides have been shown to favor the aggregation of the enzyme, and therefore to antagonize the action of DPNH[1] and the steroids.[2] It was therefore of interest to determine whether these nucleotides and L-leucine had the same site of action. Suffice it to say that when maximal stimulation by ADP of glutamate oxidation was obtained, L-leucine produced no additional effect. However, when a similar degree of activation was produced with DPN, L-leucine could cause as much stimulation as in the absence of the excess DPN.

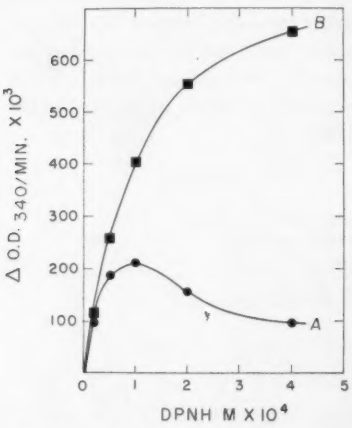

FIG. 3.—Effect of L-leucine and DPNH on the reductive amination of α-ketoglutarate. 2.5 ml reaction mixture contained 0.01 M tris buffer pH 8, EDTA 1×10^{-4} M, α-ketoglutarate 5×10^{-3} M, NH_4Cl 0.1 M, 0.0025 mg of crystalline beef liver glutamic dehydrogenase, DPNH as shown, and in curve B: L-leucine $9 \times 10^{-3} M$.

Because of the structural similarity between L-leucine and glutamate and because L-leucine has also been shown to be a substrate for the enzyme,[5] it might be supposed that the leucine and glutamate binding sites are the same. The fact that L-leucine stimulated rather than inhibited the oxidation of glutamate showed that this is not the case. The further observation that the stimulation of the GDH reaction by L-leucine was independent of the glutamate concentration (Fig. 5) also indicated that leucine and glutamate do not have a common binding site.

In our previous studies on the alanine dehydrogenase reaction[3] catalyzed by GDH, some evidence was obtained that the alanine site is different from the glutamate site. The effect of alanine on the leucine stimulation was therefore examined. Rela-

tively high concentrations of alanine (0.12 *M*) could not prevent the L-leucine activation (Table 3), which makes it appear that the sites for leucine and alanine are also different.

Discussion.—The results of these investigations indicate that the L-isomers of leucine, methionine, norvaline, and isoleucine have a pronounced effect on the struc-

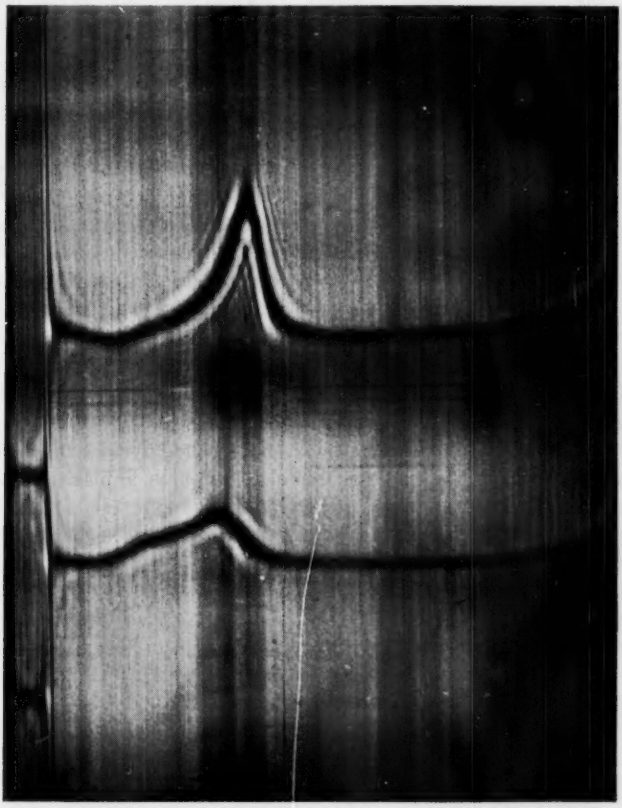

FIG. 4.—Effect of diethylstilbestrol and L-leucine on sedimentation behavior of glutamic dehydrogenase. Reaction mixture contained 0.05 *M* tris pH 8.0, EDTA 5×10^{-4} *M*, crystalline beef liver glutamic dehydrogenase 5 mg/ml, propylene glycol 2%, diethylstilbestrol 2×10^{-4} *M*, and in B: L-leucine 2.4×10^{-2} *M*. Sedimentation is from left to right for 16 minutes at a rotor speed of 59,780 rpm.

ture of crystalline glutamic dehydrogenase. These amino acids apparently favor aggregation of the subunits of the enzyme, thereby antagonizing the effects of compounds which cause its disruption. Functionally, these structural alterations are evidenced by the ability of the amino acids to stimulate the enzymic reaction and to overcome the inhibition produced by DES, DPNH, etc.

The data show that there are specific structural requirements for the amino acids which activate GDH. Since neither the fatty acid nor the α-keto acid corresponding to leucine were active, an α-amino group is necessary, and the ineffectiveness

of the D-antipode indicates a requirement for the L-configuration of the α-carbon.

All of the stimulatory amino acids had a straight chain of five atoms, and neither a longer molecule (norleucine) nor a shorter one (α aminobutyrate) affected the enzyme. The greater sensitivity of GDH to methionine than to its carbon analog, norvaline, is also interesting to note.

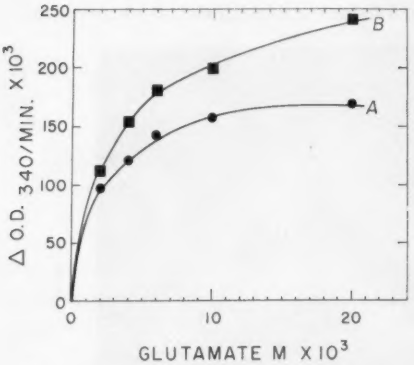

FIG. 5.—Effect of glutamate concentration and L-leucine on the glutamic dehydrogenase reaction. Experiment was done as in Figure 1 *A*, *B*, except for glutamate concentration as shown, and the presence of L-leucine 1.2×10^{-2} *M* in *B*.

Finally, attention should be directed to the influence of the branched chain in leucine, which confers upon that amino acid a 6- to 8-fold greater activity than its nonmethylated analog, norvaline.

It is, at this time, impossible to give a satisfactory chemical explanation for the action of these amino acids on the enzyme molecule, since there is no detailed information about the forces which bind the subunits together. It seems, in general, that molecules such as the steroids, phenanthridine, and the reduced pyridine nucleotides, which cause disaggregation of the enzyme, have in common a planar, nonpolar area. This suggests that adjacent polypeptide chains may interact, to some extent, by way of aromatic residues and that these interactions can be disrupted by the enzyme inhibitors. Since the molecule has been reported to dissociate on dilution,[8] it is apparent that these binding forces are not very strong.

The mechanisms by which DPN, ADP, and leucine favor protein association are also obscure, but might be due to neutralization of repulsive charges on adjacent chains. It may be relevant that when activated by ADP, GDH could no longer be stimulated by leucine which suggests that the nucleoside diphosphate and the amino acid have a common mode of action.

TABLE 3

EFFECTS OF L-LEUCINE AND L-ALANINE ON THE GLUTAMIC DEHYDROGENASE REACTION

Additive	ΔO.D.$_{340}$/min $\times 10^{-3}$	
	No leucine	L-leucine 1.2×10^{-2} *M*
None	116	184
L-alanine 4×10^{-2} *M*	120	192

Conditions were identical to those for Figure 1A, B except for addition of L-leucine and L-alanine as shown.

Aside from the chemical implications of these findings, biologically it is of interest that none of the three natural amino acids which can influence GDH structure, leucine, methione, and isoleucine, can be synthesized to any extent by mammals. Thus, the dietary intake of these amino acids could regulate the metabolism of other amino acids by influencing the state of aggregation of GDH.

Current ideas about the control of enzyme action, at least in microorganisms,

have centered around the concept of negative feed-back[9] in which a metabolic end-product can inhibit the first definitive step in its own biosynthesis. Functionally, the effect of leucine, methionine, and isoleucine on glutamate metabolism is clearly different, since these amino acids are not biosynthetically related to glutamate, but can still affect its metabolism. Furthermore, rather than producing inhibition, they stimulate glutamate metabolism.

In more general terms, the influence of leucine, described here, and of the steroid hormones[2,3] on the structure of glutamic dehydrogenase indicate that metabolic control could be mediated through changes in enzyme structure.

The leucine site which causes stimulation of the enzyme is apparently distinct from those which bind the substrates or catalyze the chemical transformations. Similarly, Frieden has proposed an activating site on GDH for DPN and ADP,[7] and an inhibitory site for DPNH.[1] GDH, therefore, carries with it not only an active center, but quite specific areas devoted to control of the enzymic reaction. It may be that the large size of this enzyme molecule, and perhaps of others, is required to accommodate not only catalytic loci but specific control sites as well.

Summary—1. L-leucine, L-methione, L-isoleucine, and L-norvaline stimulate the glutamic dehydrogenase reaction catalyzed by either a crude rat liver preparation or a crystalline enzyme from beef liver. D-leucine was ineffective.

2. The inhibition of the enzyme by DPNH, diethylstilbestrol, and other compounds can be overcome by these L-amino acids.

3. L-leucine prevents the disaggregation of the enzyme caused by diethylstilbestrol.

4. The effect of L-leucine on the kinetics of the enzymic reaction can be explained by its influence on the aggregation state of the protein.

5. These findings suggest a mechanism by which these essential amino acids can regulate general amino acid metabolism.

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*THE IDENTIFICATION OF PHYSIOLOGICAL RACES OF A FUNGAL PHYTOPATHOGEN USING STRAINS OF THE SLIME MOLD ACRASIS ROSEA**

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Physiological races of a fungal phytopathogen represent biotypes capable of attacking certain varieties of a susceptible host-species. Attempts to distinguish physiological races by other criteria have not yet been successful. On the other hand, strains of bacterial pathogens may often be identified using bacterial viruses (bacteriophage). Attempts to demonstrate a virus of a true fungus have not yet been successful.

It seemed reasonable to assume that an obligate parasite of a fungal phytopathogen might provide the means to identify its physiological races. Olive *et al.*[1] reported that the slime mold *Acrasis rosea* Olive & Stoianovitch required ingested food as its primary source of nutrients and that certain strains of the slime mold would ingest the spores of some but not other species of fungi. One "susceptible" species was *Colletotrichum lagenarium* (Pass.) Ell. & Halst., the incitant of anthracnose of cucurbits. An investigation of the "resistance" or "susceptibility" of four physiological races of this phytopathogen grown on four media and subsequently inoculated with each of six strains of *A. rosea* indicated that it was possible to identify each physiological race of *C. lagenarium*.

Materials and Methods.—Olive *et al.*[1] have reported on the origin of five strains of *A. rosea* used in this investigation; strain MW-1 was isolated by S. K. Dutta. The slime molds were "cultured" on *Rhodotorula mucilaginosa* (red yeast) which was grown at room temperature on corn meal-dextrose agar (Difco) supplemented with 0.2 per cent yeast extract. The red yeast was highly "susceptible" and was rapidly ingested by the slime molds. The migration of myxamoebae of *A. rosea* from the red yeast to the agar was detected by the appearance of an obvious haze around the red yeast culture. Small rectangles (1 × 3 mm) of agar well away from the culture were removed from the hazy area and added to the center of sporulating colonies of *C. lagenarium*. The myxamoebae migrated from the agar block to the colony. It must be emphasized that *only* sporulating colonies were inoculated. The inoculated colonies were incubated at 22°C for 5 days and then placed at room temperature in the light for an additional 5 days to determine if the spore masses of the phytopathogen had been ingested and if the slime mold had fruited.

The slime mold ingests spore masses but not the mycelium of a "susceptible" physiological race of *C. lagenarium*. The area of ingested spore masses resembled a "crater" when the surface of the colony was illuminated with incident, oblique light. If spore masses of one colony in a series of replicate plates of a "susceptible" physiological race of *C. lagenarium* were apparently not ingested, a portion of that colony which included the site of inoculation of the slime mold was transferred to a culture of red yeast. If the yeast was ingested, it was assumed that the slime mold had indeed ingested the spore masses of *C. lagenarium* but at a very slow rate.

The initial investigation was performed with one isolant of each of the four known physiological races of *C. lagenarium;* their origin was reported by Dutta *et al.*[2] Later studies involved 13 isolants supplied by N. N. Winstead who had assigned all but one to a specific physiological race.

Only those cultures of *C. lagenarium* which displayed excellent growth and sporulation on the following media were used: minimal agar,[3] potato-dextrose agar,[3] bean agar,[4] and yeast extract agar (0.5 per cent yeast extract, 2 per cent glucose, 2 per cent agar).

Colonies of *C. lagenarium* were scored on the fifth and tenth day after inoculation with the slime mold. Spore masses of "resistant" colonies were not ingested; spore masses of "susceptible" colonies were either poorly, obviously, or rapidly ingested. If the spore masses were rapidly in-

gested (highly "susceptible"), the colony exhibited a crater within 5 days and the diameter of the crater increased in size during the next 5 days. In the case of "susceptible" colonies, a crater usually did not appear within 5 days but an obvious crater was observed by the tenth day.

A brief summary of the procedures may clarify the situation with respect to strains of the slime mold (*A. rosea*), the red yeast (*R. mucilaginosa*) used as "substrate" for the slime mold, and the physiological races and isolants of the fungal phytopathogen (*C. lagenarium*). All strains of the slime mold multiplied on the red yeast; myxamoebae of the slime mold were obtained from the hazy area surrounding the culture of red yeast; sporulating colonies of the fungal phytopathogen were inoculated with myxamoebae.

Results.—The "resistance" or degree of "susceptibility" of the four physiological races of *C. lagenarium* grown on four media to the six strains of the slime mold is summarized in Table 1. All physiological races were "resistant" to the "Type" strain, regardless of the medium; one or more physiological races were "susceptible" to one or more of the other strains, depending on the medium. Certain combina-

TABLE 1

"RESISTANCE" OR DEGREE OF "SUSCEPTIBILITY" OF PHYSIOLOGICAL RACES OF *Colletotrichum lagenarium* GROWN ON DIFFERENT MEDIA TO STRAINS OF *Acrasis rosea**

Medium	Races of *C. lagenarium*	Strains of *A. rosea*					
		Type	NC-18	NE-30	NE-37	NE-68	MW-1
Minimal	1	0	0–2	0–1	0	0–1	0
	2	0	2	2	0	0–2	0
	3	0	2–4	4	0	2–4	0
	4	0	4	4	0	4	1
Yeast extract	1	0	0	0	0	0	0
	2	0	0–2	0–1	0	0–2	0
	3	0	2–4	4	1	2	0–1
	4	0	4	4	0–1	2	2
Potato-dextrose	1	0	0–2	0	0	0	0
	2	0	0–2	0–2	0	0–1	0–1
	3	0	2–4	2	0–1	2–4	2
	4	0	4	4	0–1	4	4
Bean	1	0	0–2	0–1	0	0	0
	2	0	2	2–4	0–1	0–1	0
	3	0	2–4	4	1	2	0–1
	4	0	4	4	1	2	0–1

* 0, not ingested ("resistant"); 1, poorly ingested (slightly "susceptible"); 2, obviously ingested ("susceptible"); 4, rapidly ingested (highly "susceptible").

tions of physiological race and medium gave inconsistent results with respect to "susceptibility" or "resistance" to a specific strain of slime mold. This variation is being investigated. If colonies which exhibited a very small crater had been scored as "resistant," the number of inconsistent observations would have been reduced. Strain NC-18 fruited on all "susceptible" physiological races; strain MW-1 fruited on physiological races 3 and 4 grown on potato-dextrose agar.

A scheme (Table 2) was devised to identify each physiological race of the phytopathogen by noting those combinations of medium and strain of slime mold which gave consistent results (see Table 1). The first dichotomy was established by determining the "resistance" or degree of "susceptibility" of a physiological race grown on yeast extract agar and inoculated with strain NE-30 of the slime mold. The second dichotomy required two tests to distinguish race 1 from race 2, and race 3 from race 4.

Since it was possible that four isolants of one physiological race might have given the same results as did the one isolant from each of the four known races, sporulat-

ing cultures of the 13 isolants supplied by Winstead were tested to validate the scheme presented in Table 2. The cultures were given code numbers to avoid inspired guesses. The 12 isolants of *C. lagenarium* came from different geographical sources and had already been assigned by Winstead to one of the four physiological races. All isolants were correctly identified using the procedure presented in Table 2. Isolant S94-A-1 which had not been previously identified was assigned to race 1. It will be possible to test this identification by inoculating appropriate watermelon varieties.[2]

Discussion.—The nature of the resistance of certain varieties of a susceptible host-species to physiological races of a fungal phytopathogen has not yet been satisfactorily explained.[5] Although it seems reasonable to assume that resistance may be attributed to physiological or biochemical mechanisms, direct evidence to implicate a specific physiological or biochemical difference between a susceptible and a resistant variety has not been forthcoming.

It seems reasonable to assume that differences between physiological races of a fungal phytopathogen may also have a physiological or biochemical basis. The

TABLE 2

IDENTIFICATION OF THE PHYSIOLOGICAL RACES OF *Colletotrichum lagenarium* USING STRAINS OF *Acrasis rosea*

To distinguish *C. lagenarium* races	Grown on medium	Strain of *A. rosea*	Ingestion of spore masses
1 and 2 from 3 and 4	Yeast extract	NE-30	1 and 2: none to poor. 3 and 4: rapid.
1 from 2	Minimal	NE-30	1: none to poor. 2: obvious.
	Bean	NE-30	1: none to poor. 2: obvious to rapid.
3 from 4*	Yeast extract	NC-18	3: obvious to rapid. 4: rapid.
	Bean	NC-18	3: obvious to rapid. 4: rapid.

* Race 4 was distinguished from race 3 by the *consistent* and rapid ingesting of spore masses of the former race by strain NC-18, using 10 replicates.

results presented in this report indicated that the strains of the slime mold *A. rosea* were able to distinguish physiological races of the fungal phytopathogen *C. lagenarium*. The slime mold required ingested food, in this investigation the spore masses of *C. lagenarium*, to multiply. Since the medium on which the fungal phytopathogen was grown may determine its "resistance" or "susceptibility" to a strain of the slime mold, it is possible that the biochemical status of the phytopathogen may play a significant role in its "resistance" or "susceptibility" to a strain of slime mold.[6]

The identification of isolants of the phytopathogen *C. lagenarium* as to their physiological race on the basis of their "resistance" or "susceptibility" to strains of the slime mold may be compared to typing strains of bacterial pathogens using bacteriophage. In one sense, the fungal phytopathogen was treated as a host for the slime mold. It is possible that this procedure for assigning an isolant of *C. lagenarium* to a specific physiological race without resorting to inoculating it into varieties of a susceptible host-species may be extended to other fungal pathogens.

We are indebted to L. S. Olive, Columbia University, for strains of the slime mold and to N. N. Winstead, North Carolina State College, for cultures of the fungus.

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*EVIDENCE FOR HYDROGEN BONDING IN THIOLS FROM N.M.R. MEASUREMENTS**

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The capacity of the thiol group to form hydrogen bonds is a question of some importance in organic chemistry and biochemistry. The earlier literature, which is adequately reviewed by Pimentel and McClellan,[1] indicates that the —SH group can form weak hydrogen bonds with strong donor groups. Only recently has it been shown that the sulfur atom can act as the donor[2] in hydrogen bonding involving the —SH group.

Three laboratories[3–5] have detected changes in the —SH infrared absorption which indicate hydrogen bonding in liquid thiols; the frequency shifts are small. Spurr and Byers[5] have studied the —SH stretching absorption of six mercaptans and find that the variation with concentration in carbon tetrachloride solution of the total integrated absorption coefficient can be accounted for on the basis of a monomer-dimer equilibrium.

Forsen[6] has measured the chemical shift of the —SH proton magnetic resonance of ethyl mercaptan at 40 Mc/s as a function of concentration in carbon tetrachloride. He found that the —SH signal showed a total shift of 0.38 ± 0.015 ppm toward higher field on dilution, suggesting the presence of weak but significant hydrogen bonding. The smallness of the shift and its almost linear dependence on the mole fraction of mercaptan were taken to indicate that monomers and dimers are the predominating species.

Our continuing interest in the chemistry of aminothiols[7] and in the kinetics of the oxidation of thiols to disulfides[8] has led us to extend the N.M.R. study of thiol-containing compounds. Earlier reports have been insufficient to establish the range in which the thiol resonance may appear. Meyer, Saika, and Gutowsky[9] have measured the chemical shift of the —SH group in benzenethiol and benzyl mercaptan with respect to an external water reference. Abraham, Pople, and Bernstein[10] have analyzed the spectrum of methyl mercaptan. Chamberlain[11] has listed the range of 8.4–8.8 (in τ units, ppm[12]) for the —SH group in aliphatic thiols and the value of 6.4 for benzenethiol. Only Forsen[6] has reported a dilution shift.

In order to obtain more information on the chemical shifts of the thiol proton

and to study further the formation of S → H-S hydrogen bonds, we have determined the N.M.R. spectra of *n*-propyl mercaptan, benzyl mercaptan, and benzenethiol as solutions in carbon tetrachloride. In all cases a shift of the thiol resonance on dilution has been found, showing that the observation made by Forsen[6] for ethyl mercaptan also applies to other compounds. The limits of the thiol shift in these compounds have been established and are recorded in τ units.

Experimental.—Measurements were made with a Varian V-4300 B spectrometer, operating at 60 Mc/s, and equipped with a super stabilizer and sample spinner. In most cases chemical shifts were measured by the side-band method, the mean of six to 12 determinations at each concentration being taken. The standard deviation was about 0.2 c/s. The thiol proton shifts in benzyl mercaptan and benzenethiol were measured with respect to the phenyl resonance and also with respect to

TABLE 1

DILUTION SHIFT OF THIOL RESONANCE

Compound	Chemical shift at mole fraction = 0[a] Ppm	τ	Chemical shift at mole fraction = 1 Ppm	τ	Dilution shift (ppm) [b]	[c]
n-propylmercaptan	1.34[b]	8.66	1.65[b]	8.35	0.31	
Benzylmercaptan	5.72[c]	8.49	5.44[c]	8.33	0.16	0.28
Benzenethiol	3.92[c]	6.75	3.64[c]	6.63	0.12	0.28

[a] Extrapolated value.
[b] From $(CH_3)_4Si$.
[c] From phenyl peak.

tetramethylsilane as an internal reference. Chemical shifts in *n*-propyl mercaptan were measured with respect to the previously determined fine structure of the β-CH_2 resonance, by recording this portion of the spectrum and taking the mean of six to 12 measurements. For the lower concentrations, tetramethylsilane present in about half the mercaptan concentration was used as an internal reference by the side-band method. Measurements were carried out on the lowest field member of the thiol triplet, which was not masked by other peaks at any of the concentrations used. The position of the triplet center was obtained from the coupling constant (7.6 c/s, mean of three determinations). Measurements were made at an ambient temperature of 25 ± 1°C.

Eastman Kodak chemicals were used without further purification.

Results and Discussion.—The thiol proton resonance in *n*-propyl mercaptan shows a linear dependence on concentration in carbon tetrachloride, moving to higher field on dilution (Fig. 1). In the pure liquid the thiol triplet is superimposed upon the β-CH_2 peaks. On dilution it moves to the same region as the CH_3 peaks. The triplet structure of the thiol resonance in *n*-propyl and benzyl mercaptan is retained at all concentrations, i.e., no exchange of the thiol proton can be detected.

The thiol resonances of benzenethiol and benzyl mercaptan do not show a linear concentration dependence with tetramethylsilane as reference, but do so when the phenyl peak is used as reference. Interpretation of the dilution shifts in these compounds is complicated by the aromatic ring effect, which also introduces a concentration dependence.[13] With the phenyl peak as reference, the total dilution shifts for these compounds are approximately the same as that for *n*-propyl mercaptan; with tetramethylsilane as reference the dilution shifts are smaller. Further investigation of the influence of aromatic groups is needed. The total dilu-

tion shifts, and the limits expressed in τ units, extrapolated to infinite dilution, are listed in Table 1.

A shift to higher field on dilution has been found typical of lessened intermolecular hydrogen bonding in alcohols and phenols.[14–17] The dilution shift in thiols may be interpreted in terms of hydrogen bonding of the type S → H—S—. In contrast to the large dilution shift obtained for alcohols in carbon tetrachloride (e.g., 4.57 ppm for ethanol[15]) the shifts in mercaptans are only 0.3–0.4 ppm. This is in

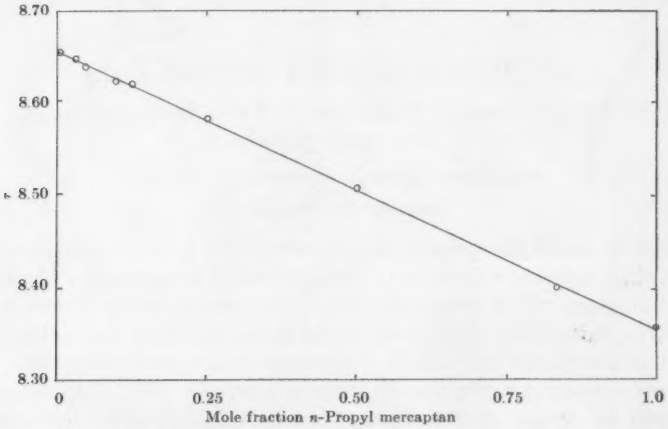

FIG. 1.—Dilution shift of the thiol proton resonance in CCl_4 solution.

accord with the small frequency shifts in the infrared. In agreement with other workers[3–6] it may be concluded that thiols are capable of exhibiting definite but weak hydrogen bonding.

Huggins, Pimentel, and Shoolery[14] have shown that a linear concentration dependence is to be expected in the case of a monomer-dimer interaction in phenols at very low concentrations. Together with the infrared evidence,[5] the linear nature of the thiol resonance shift on dilution is a strong indication that monomers and dimers are the only species present in significant concentration in mercaptan solutions.

* Aided by Contract DA-49-193-MD-2031 between the U. S. Army Medical Research and Development Command and the University of Rochester.

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A NEW TYPE OF BORON HYDRIDE, $B_{10}H_{16}$

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We wish to report the preparation, structure, and a few properties of a boron hydride which contains a direct B-B bond between B atoms neither of which has a terminal H atom. This structural feature, not present in the known hydrides,[1] B_2H_6, B_4H_{10}, B_5H_9, B_5H_{11}, B_6H_{10}, B_9H_{15}, and $B_{10}H_{14}$, probably dominates the chemistry of this new hydride and very likely gives rise to new types of reactions.

The new compound is prepared by the slow passage of B_5H_9 in an atmosphere of H_2 through an electric glow discharge between Cu electrodes. Optimum conditions for this preparation are being sought, but at present there is no difficulty in producing 2 mg/hr from one discharge tube. The compound is a solid at room temperature, stable in air, and is readily purifiable by sublimation. Its volatility is somewhat greater than that of $B_{10}H_{14}$ and considerably less than that of B_9H_{15}, and is suggestive of a B_{10} hydride distinct from the well-known $B_{10}H_{14}$. Crystals of the material, when suspended in water, rise at a noticeably greater rate than do comparable crystals of $B_{10}H_{14}$. The density of the crystals is therefore less than 0.94 gm/cm^3 and is estimated in the range 0.80–0.87 gm/cm.^{3}

An X-ray diffraction study of a single crystal has shown that the space group is $P2_1/a$, and that the unit cell parameters are $a = 8.30$ A, $b = 10.65$ A, $c = 6.08$ A, and $\beta = 111°$. Comparison of the volume of this unit cell, 492 A^3, with the molecular[2] volume of $B_{10}H_{14}$, 246 A,^{3} or the molecular volume[3] of B_5H_9, 138 A,^{3} indicates only two molecules of such a nonvolatile hydride in the unit cell. The structure is ordered, as we show in Figure 1, and hence the molecule is necessarily centrosymmetric. The projection of electron density along the short c axis suggests two tetragonal pyramidal B_5 units, each like the structural unit[3–5] in B_5H_9, connected at the apex B atoms by a single bond as shown in Figure 2. These results suggest that the molecule is H_8B_5-B_5H_8, in which the B_5H_8 units are like the B_5H_9 molecule with the apex H atom removed. The molecular formula would then be $B_{10}H_{16}$, and the corresponding calculated density of the crystal is 0.84 gm/cm.^{3} The molecular volume of 256 A^3 is between that of $B_{10}H_{14}$ and that of two B_5H_9 molecules. The agreement (Table 1) of the hkO amplitudes of X-ray scattering is summarized by the value of $R = \Sigma\big||F_o| - |F_c|\big|/\Sigma|F_o| = 0.13$. A three-dimensional X-ray diffraction study, now under way, may establish all of the H atom positions.

Spectroscopic studies, moreover, indicate a very close relation of $B_{10}H_{16}$ to B_5H_9. The infrared spectra are very similar throughout the usual range of 2 μ to 15 μ, and, in particular, the terminal B-H peak near 4.0 μ is not split in the spectrum of either compound. The B^{11} nuclear magnetic resonance spectrum of $B_{10}H_{16}$ shows a large, symmetrical, low field doublet very much like the analogous doublet in the

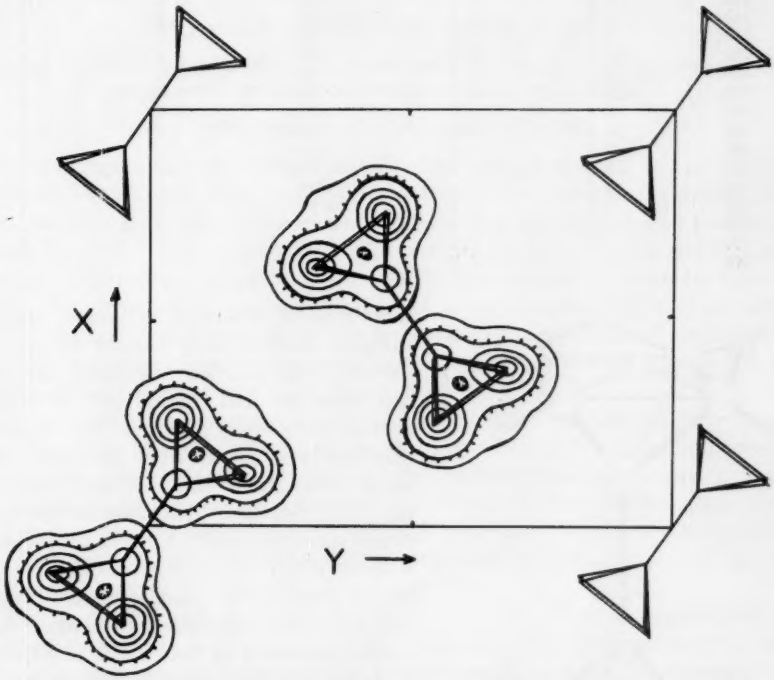

FIG. 1.—Projection of electron density along the c axis of the unit cell of $B_{10}H_{16}$. Contours are at 1, 2 (marks on the side of lower electron density), 3, 4, and 5 arbitrary units. The B-B distance between the two B_5H_8 groups is 1.74 A, the apex to basal B-B distances average to 1.76 A, and the basal to basal B-B distances bridged by H atoms average to 1.71 A, all $\pm$0.06 A. The two bridge H atoms that project on one another also occur in an electron density function, not shown here, from which the B atoms have been subtracted.

B_5H_9 spectrum.[6] As expected, the smaller high field peak is a singlet in $B_{10}H_{16}$, which has no terminal H atoms on the apical B atoms. The corresponding high field peak in B_5H_9 is, of course, a doublet, because of the apical H atom. Thus these spectra are consistent with the structure of $B_{10}H_{16}$ as indicated by the X-ray results and further suggest that the bonding of the 16 H atoms in $B_{10}H_{16}$ is very similar to the bonding of the 8 basal H atoms in B_5H_9.

Investigation of the chemical properties has only just begun. The chemical analysis shows 84.9 per cent of B and 12.0 per cent of H, in reasonable agreement with the 87.0 per cent of B and 13.0 per cent of H expected for $B_{10}H_{16}$. The

TABLE 1

OBSERVED (F_o) AND CALCULATED (F_c) AMPLITUDES OF X-RAY SCATTERING FROM $B_{10}H_{16}$

hk0	$\|F_o\|$	$\|F_c\|$	hk0	$\|F_o\|$	$\|F_c\|$
200	25.0	21.0	140	5.5	6.3
600	9.9	9.9	340	8.6	8.5
800	4.2	3.5	440	2.2	2.6
110	23.2	22.0	540	9.5	9.3
210	30.2	27.8	150	4.8	4.9
310	3.3	3.8	350	2.8	4.1
410	4.8	3.1	450	3.3	5.0
510	8.3	6.7	550	2.8	1.8
610	4.7	4.4	160	10.7	10.8
020	13.9	15.4	460	2.8	1.8
120	40.8	34.2	560	3.0	2.6
220	16.6	15.3	760	2.6	2.8
320	3.3	4.3	270	4.9	5.2
420	12.8	14.0	470	2.4	1.0
520	2.6	2.1	670	1.7	1.4
620	5.4	6.5	080	8.3	7.7
720	5.0	3.9	180	2.4	1.8
130	8.4	9.4	0.10.0	2.2	1.4
330	8.3	8.5	0.12.0	3.3	0.3
430	3.8	3.8	190	1.7	2.5
040	8.1	7.2			

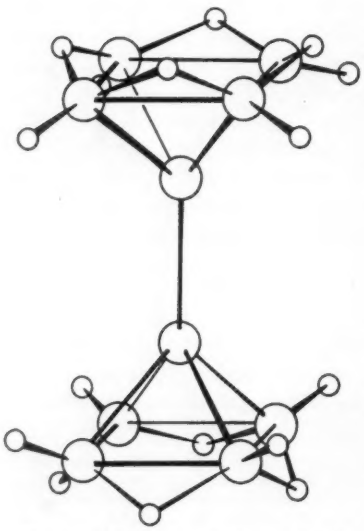

FIG. 2.—Drawing of the molecular structure of $B_{10}H_{16}$. The center of symmetry of the crystal requires the eclipsed form in the solid state, but a sizable barrier to internal rotation is not expected.

compound is insoluble in water, soluble without reaction in a variety of organic solvents such as ether, methanol, and carbon disulfide but hydrolyzes in water-acetone solutions. Many of the chemical reactions are expected to be associated with initial cleavage of the B-B bond between the B_5H_8 residues. Solutions of the compound take up Br_2 and I_2 at room temperature without evolution of H_2, possibly to give known compounds.[7] Addition to ethylene and acetylene, anticipated to produce B_5H_8 residues attached by B-C bonds to adjacent C atoms, may be a route to organic derivatives containing high B percentages. We hope that these compounds may condense to $B_{10}C_2H_{12}$ analogous to the known unreactive icosahedral $B_{12}H_{12}^{-2}$ or to derivatives,[8] if substituted ethylenes or acetylenes are employed, which are being sought as inert B^{10} carriers for neutron irradiation in tumor therapy.

We wish to thank the Office of Naval Research and the Office of Ordnance Research for support and we also thank Dr. John Baldeschwieler, who recorded the nuclear magnetic resonance spectra.

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*A THREE-COMPONENT THEORY OF SEDIMENTATION EQUILIBRIUM IN A DENSITY GRADIENT**

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In the original analysis[1] of the behavior of macromolecules and viruses in a density gradient at equilibrium in the ultracentrifuge, only two incompressible components were considered to be present. These were the polymer and the solvent which forms the density gradient. The pressure-dependent terms and the cross terms in the chemical potential which describe the chemical reactions between the polymer and the solute were neglected. It has been pointed out from theoretical considerations[2-4] that these terms are important. In experimental studies of the buoyant behavior of bovine mercaptalbumin in aqueous cesium chloride,[5] it was observed that both solvation and pressure effects are large.

In the following first-order theory of sedimentation equilibrium in a density gradient, both solvation effects and pressure dependencies are taken into account. It is shown below that a single buoyant macromolecular substance again gives rise to a Gaussian distribution of concentration. From the standard deviation of this distribution the anhydrous and the solvated molecular weight may be obtained provided certain additional ultracentrifuge, partial specific volume, and activity data are collected.

Two succeeding papers deal with the determination of the solvation[6] and the pressure-dependent parameters[7] for T-4 bacteriophage deoxyribonucleic acid. The pressure-dependent parameters for tobacco mosaic virus are also discussed.

The thermodynamic equations describing the equilibria in a three-component system in a centrifugal field are[8]

$$M_1(1 - \bar{v}_1\rho)\omega^2 r dr = \left(\frac{\partial \mu_1}{\partial m_1}\right)_{m_3} dm_1 + \left(\frac{\partial \mu_1}{\partial m_3}\right)_{m_1} dm_3; \tag{1}$$

$$M_3(1 - \bar{v}_3\rho)\omega^2 r dr = \left(\frac{\partial \mu_3}{\partial m_3}\right)_{m_1} dm_3 + \left(\frac{\partial \mu_3}{\partial m_1}\right)_{m_3} dm_1. \tag{2}$$

In these equations ρ is the density of the solution and ω is the angular velocity. The subscripts 1 and 3 refer to one of the solutes and to the macromolecule respectively. The symbols M, $\bar{v}$, μ, and m stand for molecular weight, partial specific volume, chemical potential, and molality. These differential equations are valid at constant temperature and pressure. The solvation parameter[9-11] $\Gamma \equiv -(\partial\mu_1/\partial m_3)_{m_1}/(\partial\mu_1/\partial m_1)_{m_3}$ represents the net solvation of the polymer in moles solute per mole polymer and is equal to $(\partial m_1/\partial m_3)_\mu$ by the triple product rule.

The quantity Γ is the number of moles of solute 1 which must accompany the addition of one mole of macromolecule to a very large volume of solution if this addition is to be at constant chemical potential, μ_s. Equations (1) and (2) are transformed to the more useful equations (3) and (4) upon substitution of the defined quantity Γ and the further relation[3] $(\partial\mu_1/\partial m_3)_{m_1} = (\partial\mu_3/\partial m_1)_{m_3}$. The relation between the cross terms is valid if concentrations are expressed in molalities.

$$M_1(1 - \bar{v}_1\rho)\omega^2 r dr = \left(\frac{\partial\mu_1}{\partial m_1}\right)_{m_3} dm_1 - \Gamma\left(\frac{\partial\mu_1}{\partial m_1}\right)_{m_3} dm_3; \quad (3)$$

$$M_3(1 - \bar{v}_3\rho)\omega^2 r dr = \left(\frac{\partial\mu_3}{\partial m_3}\right)_{m_1} dm_3 - \Gamma\left(\frac{\partial\mu_1}{\partial m_1}\right)_{m_3} dm_1. \quad (4)$$

Focusing attention first on the polymer, we eliminate dm_1 from equations (3) and (4).

$$[(M_3 + \Gamma M_1) - (M_3\bar{v}_3 + \Gamma M_1\bar{v}_1)\rho]\omega^2 r dr = \left[\left(\frac{\partial\mu_3}{\partial m_3}\right)_{m_1} - \Gamma^2\left(\frac{\partial\mu_1}{\partial m_1}\right)\right] dm_3. \quad (5)$$

The solvation parameter may be defined on a weight basis, $\Gamma' = \Gamma(M_1/M_3)$. Rearranging equation (5) leads to

$$M_3(1 + \Gamma')\left[1 - \left(\frac{\bar{v}_3 + \Gamma'\bar{v}_1}{1 + \Gamma'}\right)\rho\right]\omega^2 r dr = \left(\frac{\partial\mu_3}{\partial m_3}\right)_{m_1}\left[1 - \frac{(\partial\mu_1/\partial m_3)^2_{m_1}}{(\partial\mu_1/\partial m_1)_{m_3}(\partial\mu_3/\partial m_3)_{m_1}}\right] dm_3. \quad (6)$$

At the position of the maximum polymer concentration, $(dm_3/dr) = 0$. With this position defining band center, denoted here with the subscript zero, the buoyancy condition from equation (6) is[3]

$$\frac{1}{\rho_0} = \frac{\bar{v}_3 + \Gamma'\bar{v}_1}{1 + \Gamma'}. \quad (7)$$

Clearly the experimentally determined buoyant density ρ_0 is that of the solvated polymer. It should be emphasized that up to this point no assumptions have been made. The density gradient procedure provides a method for determining Γ' if $\bar{v}_1$ and $\bar{v}_3$ are known.

If we are to obtain the polymer distribution, the right side of equation (10) must be evaluated. The term, $(\partial\mu_1/\partial m_3)^2_{m_1}/(\partial\mu_1/\partial m_1)_{m_3}(\partial\mu_3/\partial m_3)_{m_1}$, which will be referred to as ϵ, is interesting. It is a general condition of stability with respect to the formation of a new phase that ϵ be less than one. To estimate an order of magnitude of ϵ, all the terms are assumed to be ideal.

$$\epsilon_i = \Gamma'^2\left(\frac{M_3}{M_1}\right)^2\frac{RT/m_1}{RT/m_3} = \Gamma'^2\left(\frac{M_3}{M_1}\right)\left(\frac{w_3}{w_1}\right), \quad (8)$$

where $w = Mm$.

At common experimental conditions for DNA and proteins in CsCl, ϵ_i takes on values between 0.2 and 8 at band center without the formation of a new phase.

Because this term is concentration-dependent, the foregoing ideality assumption predicts non-Gaussian concentration distributions.

In density gradient experiments this ideality assumption is certainly not valid, despite the very low DNA concentrations usually used. Instead, independent solvated molecules are suggested by the observed Gaussian distributions. At low polymer concentrations, the solvated molecules behave independently only at constant solute chemical potential. The assumption of independent solvated particles may be stated:

$$\left(\frac{\partial \mu_3}{\partial m_3}\right)_{\mu_1} = \left(\frac{\partial \mu_3}{\partial m_3}\right)_{m_1} + \left(\frac{\partial \mu_3}{\partial m_1}\right)_{m_3}\left(\frac{\partial m_1}{\partial m_3}\right)_{\mu_1} = \frac{RT}{m_3}. \tag{9}$$

Substituting the relations for Γ into this equation, we obtain

$$\left(\frac{\partial \mu_3}{\partial m_3}\right)_{\mu_1} = \left(\frac{\partial \mu_3}{\partial m_3}\right)_{m_1} - \Gamma^2\left(\frac{\partial \mu_1}{\partial m_1}\right)_{m_3} = \frac{RT}{m_3}. \tag{10}$$

Combining equations (5) and (6) with (10) gives an equation for the independent solvated macromolecule:

$$M_s[1 - \bar{v}_s\rho]\omega^2 r dr = \frac{RT}{m_3}\, dm_3 \tag{11}$$

where $$M_s = M_3(1 + \Gamma') \quad \text{and} \quad \bar{v}_s = \frac{\bar{v}_3 + \Gamma'\bar{v}_1}{1 + \Gamma'}.$$

The polymer introduces another interesting problem. Equation (11) has two solutions. If the solute, 1, is water, Γ' is positive; if the solute is salt, Γ' is negative. One solution yields a value of M_3 less than M_s; the other, greater. This is physically reasonable because the system cannot distinguish between dry polymer, and polymer plus an amount of solution of density $1/\bar{v}_3$ which contains Γ moles of salt per mole of polymer. To obtain the anhydrous molecular weight, Γ' must be positive. In the experimental work on DNA[6] all molalities are expressed as moles per 1000 gm of salt. In this case Γ is positive and represents moles water bound per mole polymer. M_3 becomes the molecular weight of the dry polymer.

Reexamining the value of ϵ under the assumption of independent solvated particles expressed above, we find that

$$\epsilon = \frac{\Gamma^2(\partial\mu_1/\partial m_1)_{m_3}}{(\partial\mu_3/\partial m_3)_{m_1}} = \frac{\Gamma^2(\partial\mu_1/\partial m_1)_{m_3}}{RT/m_3 + \Gamma^2(\partial\mu_1/\partial m_1)_{m_3}},$$

and ϵ will now always be less than one.

The final question to be answered by the theory is the effect of solvation on the density gradient. Upon elimination of dm_3 from equations (3) and (4), equation (12) is obtained:

$$\frac{dm_1}{dr} = \frac{[(M_1 + \gamma\Gamma M_3) - (M_1\bar{v}_1 + \gamma\Gamma M_3\bar{v}_3)\rho]\omega^2 r}{(\partial\mu_1/\partial m_1)_{m_3}[1 - \Gamma^2\gamma]} \tag{12}$$

where $\gamma = (\partial\mu_1/\partial m_1)_{m_3}/(\partial\mu_3/\partial m_3)_{m_1}$. Although hydrated water represents a very small fraction of the total water, it makes a large contribution to the water-

concentration gradient. Free water alone, however, determines the density gradient because the hydrated polymer has a density almost identical with that of the solution. The effect of the hydrated polymer on the density gradient is therefore negligible. The gradient of free water can be expressed by the equation $(dm_1/dr)_f = (dm_1/dr) - \Gamma(dm_3/dr)$. Substituting equations (5) and (12) into this expression, we obtain

$$\left(\frac{dm_1}{dr}\right)_f = \frac{M_1(1 - \bar{v}_1\rho)\omega^2 r}{(\partial\mu_1/\partial m_1)_m}. \tag{13}$$

Effects of polymer on the activity of the solvent are completely negligible at the polymer concentrations normally used. Equation (13) is the condition for equilibrium in a two-component system. It therefore does not matter that the polymer is present nor which definition of molality is used in evaluating the density gradient.

In order to obtain an expression for the polymer distribution at equilibrium, ρ, $\bar{v}_s$, and M_s are expanded about band center. $\delta = r - r_0$.

$$\rho = \rho_0 + \left(\frac{d\rho}{dr}\right)\delta, \quad \bar{v}_s = \bar{v}_{s,0} + \left(\frac{d\bar{v}_s}{dr}\right)\delta, \quad M_s = M_{s,0} + \left(\frac{dM_s}{dr}\right)\delta$$

Substituting these equations into equation (11), using the buoyancy condition $(1 - \bar{v}_{s,0}\rho_0) = 0$ and keeping only first-order terms in δ, we obtain

$$-M_{s,0}\left[\bar{v}_{s,0}\left(\frac{d\rho}{dr}\right) + \rho_0\left(\frac{d\bar{v}_s}{dr}\right)\right]\delta\omega^2 r_0 d\delta = RT\, d\ln m_3. \tag{14}$$

Integrating equation (14), we obtain a distribution of the form $m = m_0 \exp(-\delta^2/2\sigma^2)$ where σ^2 is given by equations (15) and (16).[12]

$$\sigma^2 = \frac{RT}{M_{s,0}\,\bar{v}_{s,0}\,(d\rho/dr)_{\text{eff}}\,\omega^2 r_0} \tag{15}$$

$$\left(\frac{d\rho}{dr}\right)_{\text{eff}} = \left(\frac{d\rho}{dr}\right) + \frac{\rho_0}{\bar{v}_{s,0}}\left(\frac{d\bar{v}_s}{dr}\right) \tag{16}$$

From the experimental evidence presented in references 6 and 7, $\bar{v}_s$ is known to be a function of pressure and solute activity at atmospheric pressure, a_1^0. The following equations allow the separation of these two effects and define an apparent compressibility for the solvated polymer, κ_s.

$$\frac{d\bar{v}_s}{dr} = \left(\frac{\partial\bar{v}_s}{\partial P}\right)_{a_1^0}\left(\frac{dP}{dr}\right) + \left(\frac{\partial\bar{v}_s}{\partial a_1^0}\right)_P\left(\frac{da_1^0}{dr}\right). \tag{17}$$

$$\kappa_s = -\frac{1}{\bar{v}_s}\left(\frac{\partial\bar{v}_s}{\partial P}\right)_{a_1^0}. \tag{18}$$

To express the second term on the right-hand side of equation (17) in terms of buoyant densities, the expression $1/\bar{v}_s = \rho_s$ which defines ρ_s is differentiated with respect to a_1^0. Neglecting higher-order terms, this results in

$$-\frac{1}{\bar{v}_{s,0}}\left(\frac{d\bar{v}_s}{da_1^0}\right) = \frac{1}{\rho_{s,0}}\left(\frac{d\rho_s}{da_1^0}\right). \tag{19}$$

Now $(d\rho_s/da_1^0) = (\partial\rho_0^0/\partial a_1^0)_P$. This quantity is a slope which can be experimentally determined from the buoyant density versus water activity in several aqueous cesium salt solutions.[6] This operation assumes that the anion has no effect on the buoyant density except by way of its effect on the water activity.

Upon substituting equations (17), (18), and (19) into equation (16), the effective density gradient becomes

$$\left(\frac{d\rho}{dr}\right)_{\text{eff}} = \left(\frac{d\rho}{dr}\right) - \rho_0\kappa_s\frac{dP}{dr} - \left(\frac{\partial\rho_0^0}{\partial a_1^0}\right)_P\left(\frac{da_1^0}{dr}\right). \tag{20}$$

In order to obtain a workable buoyancy condition, it is necessary to expand $\bar{v}_s$ about atmospheric pressure, P equal to zero. The activity of the solute a_1^0, and the density of the solution ρ^0 at atmospheric pressure are not independent. It is therefore possible to expand in terms of ρ^0 instead of a_1^0. The expansion is given by equation (21).

$$\bar{v}_{s,0} = \bar{v}^0{}_{s,0}\left[1 - \kappa_s P_0 - \frac{1}{\rho_0{}^0}\left(\frac{\partial\rho_0^0}{\partial a_1^0}\right)_P\left(\frac{da_1^0}{d\rho^0}\right)\left(\rho_0{}^0 - \frac{1}{\bar{v}^0{}_{s,0}}\right)\right]. \tag{21}$$

Using the relation $\rho_0 = \rho_0{}^0/(1 - \kappa P_0)$, the buoyancy condition, $\bar{v}_{s,0}\,\rho_0 = 1$, can now be written as follows:

$$\rho_0{}^0 = \frac{1}{\bar{v}_{s,0}{}^0}\left[1 - \frac{(\kappa - \kappa_s)P_0}{1 - \left(\frac{\partial\rho_0^0}{\partial a_1^0}\right)_P\left(\frac{da_1^0}{d\rho^0}\right)}\right], \tag{22}$$

where $\bar{v}_{s,0}{}^0$ is the reciprocal of the buoyant density at atmospheric pressure. Higher-order corrections throughout this discussion have been neglected. It is shown in reference 7 that the physical density gradient is given by equation (23):

$$\frac{d\rho}{dr} = \left[\frac{1}{\beta^0} + \kappa\rho^{0^2}\right]\omega^2 r. \tag{23}$$

The complete effective density gradient is obtained by substituting equation (23) into equation (20). Higher-order corrections are again neglected and the a_1^0 dependence is expressed in terms of the density at atmospheric pressure as a concentration variable. The substitution $dP/dr = \rho\omega^2 r$ (10) has also been made.

$$\left(\frac{d\rho}{dr}\right)_{\text{eff}} = \left[\frac{1}{\beta^0} + \frac{(\kappa - \kappa_s)\rho^{0^2}}{1 - \left(\frac{\partial\rho_0^0}{\partial a_1^0}\right)_P\left(\frac{da_1}{d\rho^0}\right)}\right]\left[1 - \left(\frac{\partial\rho_0^0}{\partial a_1^0}\right)\left(\frac{da_1^0}{d\rho^0}\right)\right]\omega^2 r. \tag{24}$$

All the terms of this equation can be experimentally determined. The quantity

$$(\kappa - \kappa_s)/[1 - (\partial\rho_0^0/\partial a_1^0)_P(da_1^0/d\rho^0)] = \Psi$$

can be determined by studying the pressure dependence of the buoyant density, as can be seen from equation (22). The quantity $(\partial\rho_0^0/\partial a_1^0)_P$ is the slope of buoyant density versus solute activity at P equals zero. The quantity $(da_1^0/d\rho^0)$ is the slope of solute activity versus density for the salt solution at P equal zero.

Upon multiplying as indicated in equation (24), and recalling that the composition density gradient $d\rho^0/dr = \omega^2 r/\beta^0$, we find that the effective density gradient is

$$\left(\frac{d\rho}{dr}\right)_{\text{eff}} = \left(\frac{d\rho^0}{dr}\right) + (\kappa - \kappa_s)\rho_0^2\omega^2 r - \left(\frac{\partial\rho_0^0}{\partial a_1^0}\right)_P \left(\frac{da_1^0}{d\rho^0}\right)\left(\frac{d\rho^0}{dr}\right) \tag{25}$$

or

$$\left(\frac{d\rho}{dr}\right)_{\text{eff}} = \left(\frac{d\rho^0}{dr}\right) - \left(\frac{\partial\rho_s^0}{dr}\right)_P + (\kappa - \kappa_s)\rho_0^2\omega^2 r.$$

For convenience, $(\partial\rho_s^0/\partial r)_P$ is set equal to $(\partial\rho_0^0/\partial r)_P$, defined as above.

The effective density gradient is now seen to be the composition density gradient dimmished by the polymer density gradient associated with solvation changes and enhanced by an effective compression density gradient. This latter density gradient is the result of the difference in compressibilities of soivent and the solvated polymer. The effective density is thus in the appropriate form for the physical situation.

The solvated macromolecules are everywhere in the band in equilibrium with the layer of solution perpendicular to the field. In each layer, the hydrated macromolecules are differently solvated and in addition differently compressed.

Appendix.—It can be shown that the assumption that $(\partial\mu_3/\partial m_3)_{\mu_1} = RT/m_3$ is consistent with the idea that Γ is independent of polymer concentration at constant μ_1. Partial differentiation at constant μ_1 of the expression defining Γ, $(\partial\mu_3/\partial m_1)_{m_3} = -\Gamma(\partial\mu_1/\partial m_1)_{m_3}$ with respect to m_3 gives

$$-\frac{\partial}{\partial m_3}\left[\left(\frac{\partial\mu_3}{\partial m_1}\right)_{m_3}\right]_{\mu_1} = \left(\frac{\partial\Gamma}{\partial m_3}\right)_{\mu_1}\left(\frac{\partial\mu_1}{\partial m_1}\right)_{m_3} + \Gamma\frac{\partial}{\partial m_3}\left[\left(\frac{\partial\mu_1}{\partial m_1}\right)_{m_3}\right]_{\mu_1};$$

$$\frac{\partial}{\partial m_3}\left[\left(\frac{\partial\mu_3}{\partial m_1}\right)_{m_3}\right]_{\mu_1} = \frac{\partial}{\partial m_1}\left[\left(\frac{\partial\mu_3}{\partial m_3}\right)_{\mu_1}\right]_{m_3} = \frac{\partial}{\partial m_1}\left(\frac{RT}{m_3}\right)_{m_3} = 0;$$

$$\frac{\partial}{\partial m_3}\left[\left(\frac{\partial\mu_1}{\partial m_1}\right)_{m_3}\right]_{\mu_1} = \frac{\partial}{\partial m_1}\left[\left(\frac{\partial\mu_1}{\partial m_3}\right)_{\mu_1}\right]_{m_3} = 0.$$

Therefore $(\partial\Gamma/\partial m_3)_{\mu_1}(\partial\mu_1/\partial m_1)_{m_3} = 0$. For stability $(\partial\mu_1/\partial m_1)_{m_3} > 0$. Therefore, $(\partial\Gamma/\partial m_3)_{\mu_1} = 0$.

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‡ Contribution No. 2705.

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*THE NET HYDRATION OF T-4 BACTERIOPHAGE DEOXYRIBONUCLEIC ACID AND THE EFFECT OF HYDRATION ON BUOYANT BEHAVIOR IN A DENSITY GRADIENT AT EQUILIBRIUM IN THE ULTRACENTRIFUGE**

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In an earlier publication[1] the buoyant density of DNA was shown to be a monotonic function of water activity. This was shown in two ways: (1) The buoyant density of DNA was studied in solutions containing LiBr-CsBr mixtures. (2) The buoyant density of DNA was evaluated in different cesium salt solutions. Activity data for some of the solutions were not available. Isopiestic measurements have now been carried out, and the results are presented in this paper.

The conclusion reached earlier that DNA is extensively hydrated in dilute salt solutions is corroborated. The activity-dependent terms in the expression for the effective density gradient discussed in the preceding paper[2] have been evaluated at the buoyant density of DNA for each of the salts and are tabulated.

Materials.—Cesium chloride: The CsCl was supplied by Maywood Chemical Co. It was treated with activated charcoal which had been washed three times with distilled water, and was then crystallized three times from a water solution saturated at its boiling point. The optical density for 1 cm at 260 mμ, OD^{260}, of a $\rho = 1.7$ solution of the final material was 0.025.

Cesium bromide: The CsBr was supplied by A. B. Mackay, Inc. It was used as supplied by the manufacturer.

Cesium iodide: The CsI was purchased from the Kawecki Chemical Co. It was used as supplied except that an insoluble brown material was centrifuged from a 45 weight per cent solution of the salt in water. The stock solution had an OD^{260} of infinity and a density of 1.55.

Cesium sulfate: The Cs_2SO_4 was supplied by the Kawecki Chemical Co. Insoluble matter was separated from a 50 weight per cent solution by centrifugation. Because the solution had a pH of 2.9, it was titrated with cesium carbonate solution to pH 9 after boiling. The salt was then precipitated with 5 volume parts anhydrous methanol. The precipitate was separated from the solution by centrifugation and rinsed five times with methanol. The methanol was evaporated from the salt in a vacuum desiccator. The stock solution of this material had a density of 1.66, a pH of 7.0, and an OD^{260} of 0.08.

Cesium acetate: The CsAc was made by titrating a 50% cesium carbonate solution with glacial acetic acid. The Cs_2CO_3 was supplied by the Kawecki Chemical Co. After boiling, the titrated solution had a pH of 7. Insoluble material was separated by centrifugation. The solution was evaporated until its boiling point at atmospheric pressure reached 190°C. The cooled residue was taken up in two weight parts anhydrous methanol and evaporated again to 190°C. The residue was dissolved in four weight parts anhydrous methanol, and the solution was evaporated until it was about 50 weight per cent CsAc. Eight volume parts of anhydrous ethyl ether were then added to the solution and the CsAc precipitated. The product was filtered on paper and dried in a vacuum desiccator. Insolubles were separated by centrifugation. The stock solution of this salt had a density of 2.020, a pH of 7.6 and an OD^{260} of 0.78.

Cesium formate: Cesium carbonate was titrated with formic acid. The solution was evaporated until its boiling point reached 160°C. The cooled residue was dissolved in about two weight parts methanol and evaporated to 160°C. This step was repeated and the residue again dissolved in two weight parts anhydrous methanol. To this solution 20 parts ethyl ether were added. A small semi-solid phase formed which was separated from the bulk ether and repeatedly rinsed with ether. The solid was dried in a vacuum desiccator. A stock solution of this cesium formate had a density of 2.105, a pH of 6 to 7 and an OD^{260} of 0.55.

Cesium selenate: This salt was prepared by heating stoichiometric amounts of selenious acid and cesium carbonate in a porcelain crucible.[3] A 50% solution of the Cs_2SeO_4 had a pH of 11, indicating a small excess of Cs_2CO_3. The solution was titrated to pH 9 with nitric acid. The final stock solution had a density of 1.46, an OD^{260} of 0.43, and a nitrate ion concentration of less than 0.1*M*.

Lithium silicotungstate: Lithium carbonate was added to a 50% silicotungstic acid solution to a pH of 4.5. Carbon dioxide was removed by evacuation of the solution. The stock solution had a density of 1.63 and an OD^{260} of infinity.

All other chemicals not given a specific source were standard reagent grade materials.

The emission spectrographic analyses of most of the cesium salts used in this work are given in Table 1. Although in most cases there are cationic impurities present at a concentration of about 1%, the activity trends observed are too large to be explained by these analyses. In addition, there is no obvious parallel between the analyses and the buoyant densities in the salt solutions. It cannot be said, however, that there are not small errors in buoyant densities because of these cationic impurities.

TABLE 1

EMISSION SPECTROGRAPHIC ANALYSIS OF CESIUM SALTS

	Weight Parts per Million								Weight Percent			
	Ba	V	Ca	Mg	Ti	Zr	Al	Si	Fe	Na	K	Rb	Li
Cesium Chloride	t	20	30	—	10	2	50	30	—	0.01	0.005	—	—
Cesium Formate	15	80	60	—	60	6	150	80	t	0.01	0.2	0.8	0.002
Cesium Acetate	11	50	50	—	40	6	100	40	—				
Cesium Sulfate	2	30	70	t	20	4	80	15	t	0.01	0.2	0.5	0.002
Cesium Carbonate	6	20	25	—	—	2	60	50	t	0.01	0.3	1.0	0.004
Cesium Bromide	t	30	50	—	15	2	80	10	—	0.05	0.03	0.5	0.005

t = trace. — = no detectable amount. Blank indicates no measurement made.

Procedures.—The method used for evaluating buoyant density was to determine band position in two solutions of the same salt. Solution densities were selected so that one solution would be too dense and the other too light. It was then assumed that the gradient and the buoyant density in both solutions were the same. The following equations apply to this situation:

$$\rho_0 = \rho_{e,1} + \left(\frac{d\rho}{dr}\right)(r_{0,1} - r_{e,1})$$

$$\rho_0 = \rho_{e,2} + \left(\frac{d\rho}{dr}\right)(r_{0,2} - r_{e,2}) \qquad (1)$$

$$\left(\frac{d\rho}{dr}\right) = \frac{\rho_{e,2} - \rho_{e,1}}{(r_{0,1} - r_{e,1}) - (r_{0,2} - r_{e,2})},$$

where r_0 is the banding position, r_e is the isoconcentration point,[4] and ρ_0 and ρ_e are the densities at the respective points. The subscripts 1 and 2 refer to the different solutions, and $d\rho/dr$ is the density gradient. The position r_e was taken to be at the center of the liquid column of each cell. The density ρ_e was measured with a calibrated 0.3 milliliter micropipette to an accuracy of ± 0.001 gm/cc. This treatment neglects the effects of pressure and assumes that the density gradient is constant. Corrections to the center of the cell were generally small, so errors were small. Densities are estimated to be accurate to $\pm 0.3\%$.

The T-4 bacteriophage DNA was liberated from a phage stock with guanidinium chloride as described.[5] The DNA was not precipitated with alcohol for the solvation studies. The stock solutions of DNA were in the pH 9 buffer,[5] and all stock salt solutions with the exception of the lithium silicotungstate solution were diluted with the same buffer to the appropriate density. The DNA concentrations employed for the density gradient runs were 2 to 20×10^{-6} gm/cc. Except in cesium acetate, all band positions were determined using the schlieren optical system. In cesium acetate, the band was too broad to be seen with the schlieren system, so the ultraviolet optics were used. Equations for refractive index versus density of several of the salt solutions are listed in Table 2. These were experimentally determined for the density ranges listed. They should be considered approximate because the salt solutions were diluted with pH 9 buffer and therefore

contain small amounts of sodium chloride. The curves in addition were mostly determined from only four points. They are useful, however, as approximate guides.

Because the silicotungstate ion is unstable in basic solutions, the lithium silicotungstate solution was diluted with 0.1 *M* sodium acetate pH 4.7.

Equation (22) in reference 2 shows that if the effect of a_1^0 on ρ_0 is studied, it should be done at $P = 0$. In practice, the effect of water activity on buoyant density is so much larger than the pressure effect under experimental conditions that the pressure has been ignored in the solvation studies.

TABLE 2

REFRACTIVE INDEX VERSUS DENSITY FOR VARIOUS CESIUM SALTS

Salt	Equation	Density range
Cs_2SO_4	$n_D^{25} = 0.0730\ \rho_4^{25} + 1.2646$	1.40–1.70
Cs formate	$n_D^{25} = 0.0728\ \rho_4^{25} + 1.2688$	1.72–1.82
Cs acetate	$n_D^{25} = 0.0930\ \rho_4^{25} + 1.2485$	1.80–2.05
Cs_2SeO_4	$n_D^{25} = 0.0827\ \rho_4^{25} + 1.2547$	1.38–2.00

Because osmotic coefficient data were not available for several of the salts studied, isopiestic measurements were made to determine water activity. The apparatus used is shown in Figures 1 and 2. About 1 ml of solution was placed in the two platinum crucibles, one containing the cesium salt solution, the other a solution of sulfuric acid. The copper block in which the crucibles

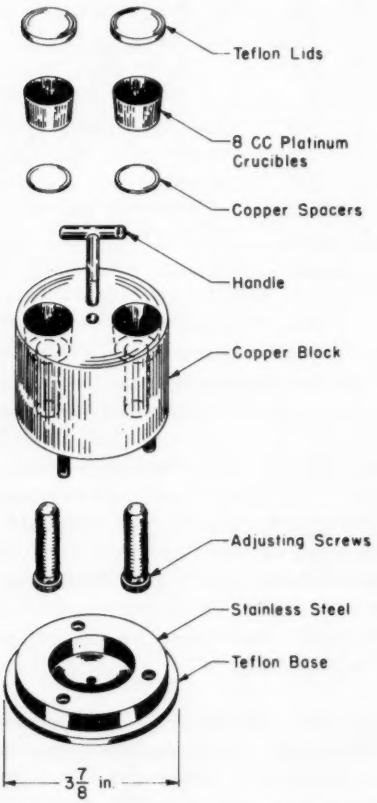

FIG. 1.—Copper block assembly for the isopiestic measurements.

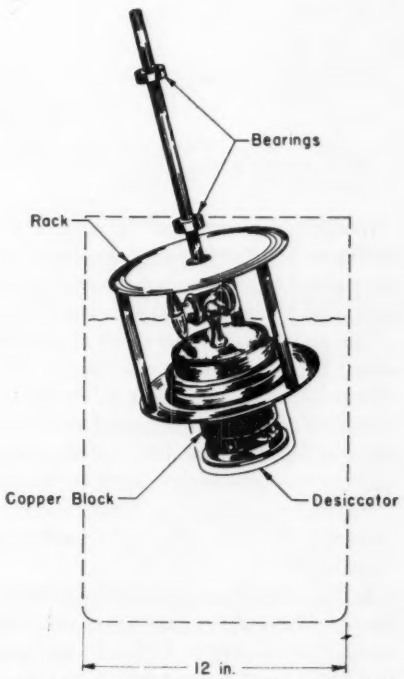

FIG. 2.—Assembled apparatus for isopiestic measurements.

were set assured thermal equilibrium between the solutions. The block was then placed in the glass desiccator. The system was evacuated with a water aspirator, and the entire assembly was rotated in a 25°C water bath for twenty-four hours with the axis of rotation about 5° off the vertical axis.

The rotation at a rate of three revolutions per minute kept the solutions constantly mixed. The weight per cent of the equilibrated sulfuric acid solution was determined refractometrically at 15°C on a Zeiss Abbé Model C-5032 Refractometer, using the data of Veley and Manley[6] for the sodium D line. Water activities were then determined from a plot of a_w at 25°C against weight per cent sulfuric acid drawn from data in Robinson and Stokes[7] and Harned and Owen.[8] The densities of the salt solutions were determined as described previously.

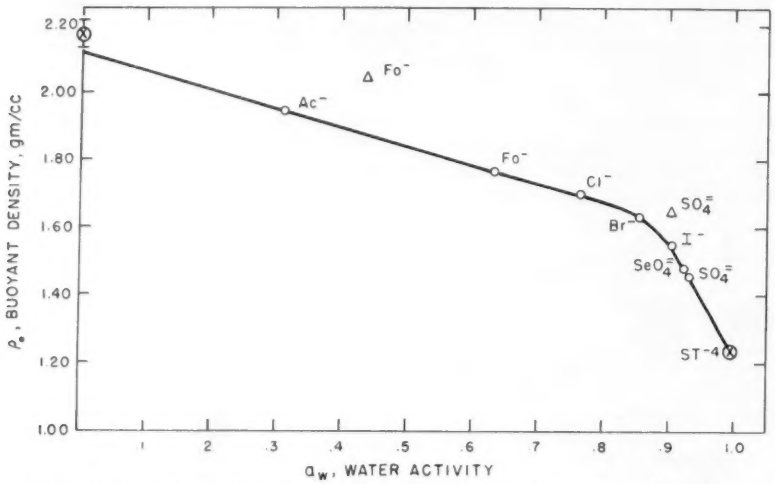

FIG. 3.—Buoyant density in various cesium salt solutions: Ac^- is acetate; Fo^-, formate; ST^{-4}, silicotungstate. The symbol ○ refers to DNA; △, to RNA; and ⊗ to CsDNA corrected from the lithium salt.

Results.—The relation between water activity and buoyant density is shown in Figure 3. The buoyant densities[9] of T-4 DNA in different cesium salt solutions are plotted against water activity calculated from data in Robinson and Stokes[7] or measured by the isopiestic method.

The point at a_w equal to zero in Figure 3 was calculated from the linearly extrapolated LiDNA value, as described.[1] The value obtained from this calculation was 2.18 ± 0.04 gm/cc. The intercept, $\rho = 2.12$, of Figure 3 is outside of the estimated margin of error. This is caused either by the assumptions made to arrive at the 2.18 figure or by the possibility that the extrapolation should not be linear. The agreement to 3 per cent does suggest there is no error in concept.

Figure 3 also shows two RNA points. The cesium formate density was taken from Davern's thesis.[10] The cesium sulfate point was measured at pH 5.5 using ultraviolet optics.[11]

LiDNA has been banded in lithium silicotungstate at pH 4.7. The buoyant density of the DNA in this solution was 1.138 gm/cc. This value was corrected to the cesium salt of DNA by the procedure described in reference 1. In addition DNA has been banded in sodium, potassium, and ammonium silicotungstate. In all cases, the buoyant densities were equal to or lower than the above value in

lithium silicotungstate solution. This clearly shows that the large amount of hydration of DNA is not restricted to the lithium form.

Figure 4 shows Γ' and Γ_n plotted against a_w. Γ_n is the solvation parameter in moles water per mole average nucleotide. Γ' was calculated using the following equation:

$$\frac{1}{\rho_0} = \frac{v_3 + \Gamma' v_1}{1 + \Gamma'}. \tag{2}$$

The specific volume of the solvated water, v_1, was taken to be one. The specific volume v_3 was taken to be the extrapolated specific volume of CsDNA or LiDNA at $a_w = 0$. The value of $1/v_3$ used for CsDNA was 2.12; for LiDNA it was 1.783 gm/cc. The Γ' scale in Figure 4 applies only to the CsDNA points. It appears from the LiDNA points that the lithium form of DNA binds water more readily than the cesium form at low water activities. This could be real or just the result of an incorrect choice of v_1. The extension of this same plot to $\Gamma_n = 50$ for CsDNA assumes that the lithium form and cesium form of DNA solvate equally. Although a difference is suggested, it is assumed to be small. The triangle on Figure 4 refers to the first point in the LiBr-CsBr system where the DNA binds an appreciable amount of cesium ion. It would be expected to be low, as it is.

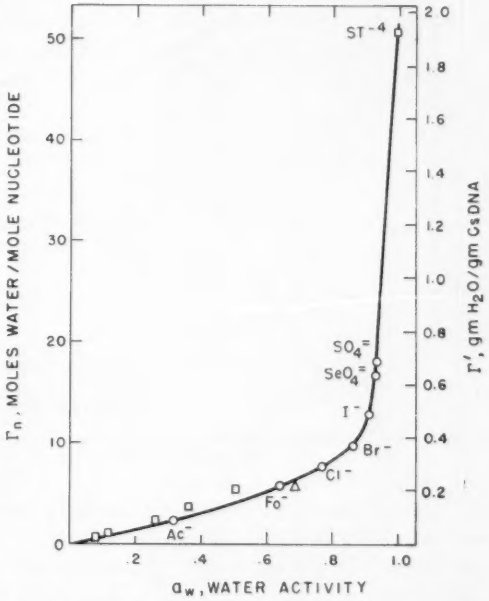

FIG. 4.—The net hydration of T-4 bacteriophage DNA. The symbol ○ refers to CsDNA; □, to LiDNA. Δ is explained in the text.

Figure 5 shows the results of the isopiestic measurements. The curves for cesium acetate, formate, and selenate, and lithium silicotungstate were determined by the method previously described. The cesium chloride curve was calculated from osmotic coefficient data in Robinson and Stokes,[7] using the relation

$$Wt\%\ \mathrm{CsCl} = 137.48 - 138.11\left(\frac{1}{\rho_4^{25}}\right)$$

for CsCl solutions. The two circles on the CsCl plot represent activities evaluated as a check on the system. These points were started much farther from equilibrium than any of the points evaluated on the unknown solutions. The two CsCl determinations, therefore, constitute a check that equilibrium was reached in all cases. The estimated accuracy of the activities determined is ±0.002.

Figure 6 shows the solution densities plotted against calculated water activities for CsBr, CsI, and Cs_2SO_4 solutions. The density-weight fraction data used in the calculations were found in ICT.[13]

Discussion.—The solvation parameter measured is a net hydration.[2] In molecular terms, it can be defined as the minimum weight of water from which the salt is excluded by the presence of the DNA. In this case of complete exclusion, the

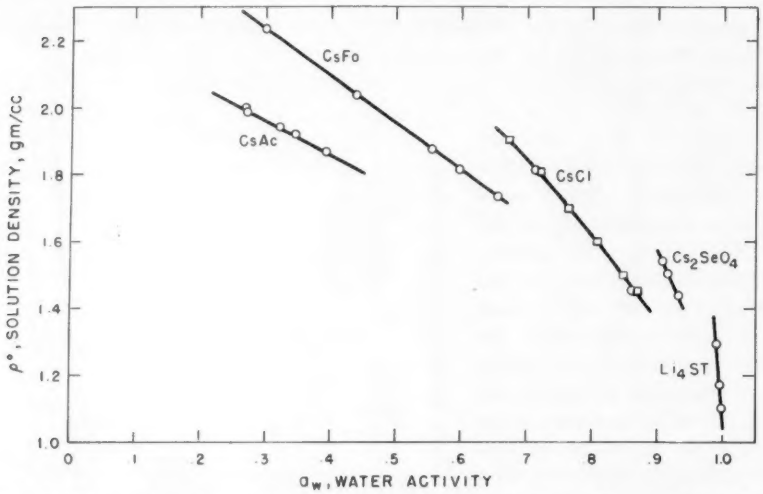

FIG. 5.—Measured water activities in various cesium salt solutions. The symbol ○ refers to measured points; □, to calculated points.

thickness of the water shell about the DNA molecule in the lithium silicotungstate solution is 11 to 12 Å. This corresponds to approximately four layers of water molecules. In the calculation, the DNA was regarded as a cylinder of radius 10 Å and density of 2.12. If the salt is not totally excluded from the hydrate water, the extent of the salt-poor hydrate will be larger. Calculating further with a 3.4 Å average distance between base planes and a Cs nucleotide residue weight of 477, we find that the volume in the grooves corresponds to 0.17 cc per gram of CsDNA. This is about 60 per cent of the net hydration in the CsCl solution and twice as large as the net hydration in Cs acetate solution.

The analysis presented in this treatment of the net hydration of DNA has assumed that water activity alone determines the extent of hydration. There is the possibility that the various salts affect the net solvation to different extents because of different sizes and charges. The charge is not believed to be important in concentrated salt solutions. The effect of size has been considered by many previous workers,[14] and calculations have been made[15] based upon free volume theory[16] with the assumption that interaction energies between solvent and solute are negligible. This assumption is a poor one in the case of the ionic solutions employed here. The formation of an ion dilute region around the DNA molecule because of a solute-size effect will be counteracted by attractive forces between water and the ions. Small energy differences between the water in the bulk solu-

tion and in the hypothetical phase associated with size differences would result in the elimination of such a phase by ion accumulation. We assume therefore that the observed net solvations may be completely described by the chemical potential of the water and the adsorbent properties of the DNA.

The large changes in buoyant density in the LiBr-CsBr mixtures at low CsBr concentrations cannot be explained by a size-exclusion mechanism because the density increments of the CsBr in the bulk solution are less than the changes in the buoyant density observed. Finally the agreement between the net solvation, Γ', in Figure 4 for DNA in CsCl solution and the value calculated from the density difference between ^{14}N and ^{15}N DNA with the effective density gradient provides an internal check on the effective gradient in a single salt solution. It is shown below that the effective density gradient gives a good estimate of the solvation.

It should be noted that the silicotungstate point in Figure 4 is subject to larger error than all other points in this figure. It is the result of a conversion, as explained earlier, from LiDNA to CsDNA. Also it was obtained at a lower ion concentration, approximately 0.5 molar, than the other data. Finally, the silicotungstate ion is the largest of the anions used and has a hydrated radius of 5.6 Å.[17]

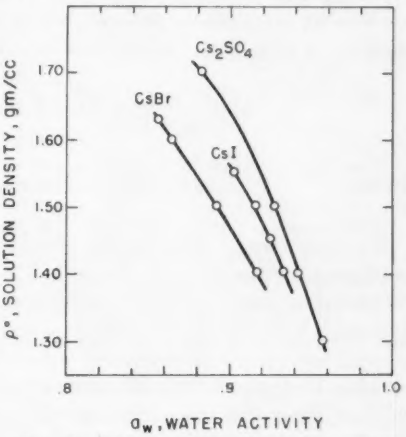

FIG. 6.—Water activities in various cesium salt solutions calculated from data in reference 7.

In the case that salt is totally excluded and the size-exclusion principle is not significant, some structure of water around the DNA must be responsible for the exclusion. This structure results from the interaction of DNA and water. Salt-poor solvates can be expected to occur for statistical or labile structures[18] of ordered water induced by the DNA.

That a water shell is necessary to the existence of the B form of DNA, in which the bases are perpendicular to the axis of the helix, is known from crystallographic data. In a recent summary,[19] X-ray diffraction patterns of the B form of Na, K, and RbDNA were found in unstretched fibers at 92% relative humidity, water activity 0.92, but not at 75% relative humidity. The data of Emanuel[20] indicate moreover that structural transitions occur in salt solutions. His data for NaCl and NaBr solutions of DNA have been successfully correlated with water activity.[21] In the correlation it was noted that LiDNA does not undergo such transitions in LiCl solutions and that transitions do not occur in LiDNA fibers at comparable water activities.

The density gradient method offers possibilities of further elucidation of the structure of the solvate layer. In order to calculate Γ, a value of $v_1 = 1$ was assumed in equation (2). If an alternative relation

$$\frac{1}{\rho_0} = \frac{\bar{v}_3 + \Gamma' \bar{v}_1}{1 + \Gamma'} \tag{3}$$

is used and the $\bar{v}_3$ and $\bar{v}_1$ are measured, Γ' can be evaluated without ambiguity and v_1 calculated from equation (2). This quantity is the average specific volume of solvate water. For this calculation v_3, which is the reciprocal of the anhydrous DNA density, is taken to be the reciprocal of the extrapolated buoyant density at $a_w = 0$.

Equations (2) and (3) are equal because $\bar{v}_3 = v_3 + \Gamma'\Delta v_1$ and $\bar{v}_1 = v_1 - \Delta v_1$, where Δv_1 is the change in the specific volume of water upon addition to the solvate layer.

In addition, if $\bar{v}_3$ and $\bar{v}_1$ are evaluated at a temperature other than 25°C in the various salt solutions and the buoyant densities are determined at that temperature, ΔH and therefore ΔS for the hydration reaction can be determined as a function of Γ'. Such information would aid greatly in determining the structure of the hydrate layer.

The apparent compressibility, $\kappa_s = -1/\bar{v}_s(\partial\bar{v}_s/\partial P)_{a_1^0}$, is evaluated at constant water activity at atmospheric pressure, which for a single salt solution implies constant molality of all species. κ_s may now be written

$$\kappa_s = \frac{1}{\bar{v}_3 + \Gamma'\bar{v}_1}\left[\bar{v}_3\bar{\kappa}_3 + \Gamma'\bar{v}_1\bar{\kappa}_1\right] - \frac{\bar{v}_1 - \bar{v}_3}{(1+\Gamma')^2}\rho_0\left(\frac{d\Gamma'}{dP}\right) \tag{4}$$

where

$$\bar{\kappa} = -\frac{1}{\bar{v}}\left(\frac{\partial\bar{v}}{\partial P}\right)_m.$$

The first term on the right side of equation (4) is the conventional compressibility coefficient for the solvated species. The second accounts for any volume changes which accompany changes in solvation caused by pressure changes. κ_s can be evaluated from the dependence of buoyant density on pressure.[22] The second term in equation (4) can be evaulated with little error if $(d\Gamma'/dP)$ is known. This derivative is related to the difference in the average specific volume of water in the hydrate layer and the partial specific volume of water in the solution. In CsCl the density shift associated with this term has been calculated to be $\Delta\rho_0^0 = (4 \times 10^{-4}) \times (v_1 - \bar{v}_1)\Delta P$, where P is in atmospheres.

The density shift was evaluated in the following way. Buoyant density was plotted against chemical potential of water in the solvate layer. At equilibrium, this potential is the same for nonsolvate and solvate water. The density shift, $\Delta\rho_0^0$, is the product of the slope of the above relation and the terms, $18(v_1 - \bar{v}_1)\Delta P$ and $1/0.82$.

The effective density gradient was shown in reference 2 to be

$$\left(\frac{d\rho}{dr}\right)_{\text{eff}} = \left\{\frac{1}{\beta^0} + \frac{(\kappa - \kappa_s)\rho^{0^2}}{1-\alpha}\right\}(1-\alpha) \tag{5}$$

where

$$\alpha = \left(\frac{\partial\rho_0^0}{\partial a_1^0}\right)_P\left(\frac{da_1^0}{d\rho^0}\right).$$

The quantity α can be evaluated for the several Cs salts considered here. The derivatives, $(\partial\rho_0^0/\partial a_1^0)_P$, are the slopes at each of the points in Figure 3. The derivatives $(da_1^0/d\rho^0)$ are the reciprocals of the slopes of solution density versus water activity at the points of intersections of these plots with the curve in Figure

3. Figure 7 displays these intersections. It also demonstrates that because the absolute values of the slopes for the salt solutions are greater than those for CsDNA, the quantity α is in all these cases less than one. This is a necessary condition for stable equilibrium at the intersection. At water activities above the point of

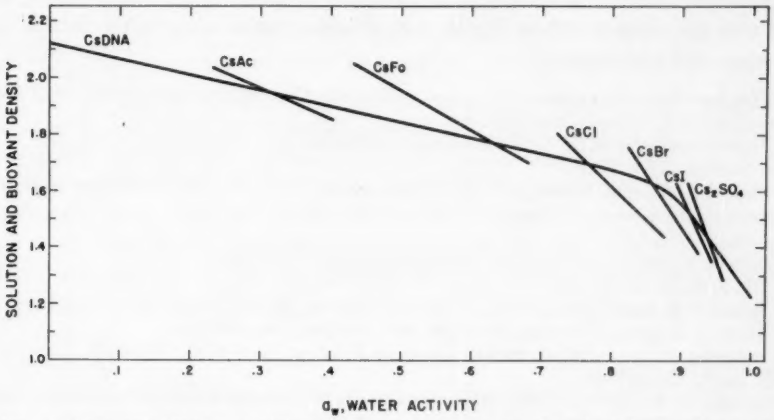

FIG. 7.—Buoyant density of cesium DNA and solution density of various cesium salt solutions superimposed to illustrate points of equilibrium.

equilibrium the solution must have a density less than that of the solvated DNA; correspondingly at lower water activities the density of the solution must be greater than that of solvated DNA for stable equilibrium to exist.

The individual values and the products of these slopes are given in Table 3.

For DNA in CsCl solutions the second term in the braces was evaluated[22] as the quantity $\psi\rho^{0^2}$. The effective density gradient in this system is $0.82\left(\frac{1}{\beta^0}\right)\omega^2 r$.

TABLE 3

ACTIVITY-DEPENDENT CORRECTION TERMS FOR VARIOUS CESIUM SALTS AT THE BUOYANT DENSITY OF DNA

Salt	$(d\rho^0/da_1^0)$	$(\partial\rho_0^0/\partial a_1^0)P$	$(\partial\rho_0^0/\partial a_1^0)P\ (da_1^0/d\rho^0)$
Cs Acetate	−1.04	−0.56	0.54
Cs Formate	−1.41	−0.56	0.40
CsCl	−2.32	−0.56	0.24
CsBr	−3.8	−1.0	0.26
CsI	−5.8	−3.2*	0.55
Cs_2SO_4	−7.0	−3.2*	0.46
Cs_2SeO_4	−3.8	−3.2*	0.84

* Although the slope of the curve in Figure 3 can be determined to an accuracy of at least ±0.1, the drawing of the best curve through these points is in question. These numbers and the corresponding numbers in the last column should be considered accurate to only ±20%.

Determination of Solvation from the Separation of Isotopically Labelled DNA's.—If we refer to the data of Meselson and Stahl,[23] the solvation may be calculated from the observed shift in the density of DNA upon total ^{15}N labeling. If this calculation is performed with the composition density gradient, $\left(\frac{d\rho}{dr}\right) = \left(\frac{1}{\beta^0}\right)\omega^2 r$, the DNA

appears to be unhydrated. The DNA, however, reacts to the entire effective density gradient, equation (5). Upon recalculating with the effective density gradient, $\left(\frac{d\rho}{dr}\right) = 0.82\left(\frac{1}{\beta^0}\right)\omega^2 r$, the solvation Γ' is found to be 0.22 ± 0.05. This number agrees well with the value of 0.28 on Figure 4, considering the accuracy of the data in the Meselson and Stahl paper.

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† Predoctoral Fellow of the National Science Foundation.

‡ Contribution No. 2705.

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*THE EFFECTS OF PRESSURE ON THE BUOYANT BEHAVIOR OF DEOXYRIBONUCLEIC ACID AND TOBACCO MOSAIC VIRUS IN A DENSITY GRADIENT AT EQUILIBRIUM IN THE ULTRACENTRIFUGE**

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In the original analysis[1] of the behavior of macromolecules and viruses in a buoyant density gradient at equilibrium in the ultracentrifuge, all components were assumed to be incompressible. As a few hundred atmospheres are normally generated in the liquid, it is to be expected that previous results based on the assumption of incompressibility require re-examination.

Several consequences arise on consideration of previously ignored pressure-dependent terms. It is shown below that these may be separately considered.

(*a*) There is a redistribution of solute with respect to solvent in the binary medium.

(*b*) The solution is compressed with no change in molality in each of the thin layers perpendicular to the centrifugal field in the liquid column. This compression adds a *compression density gradient* to the *composition density gradient.*

(*c*) The banding macromolecular species is compressed and moves to a new neutrally buoyant solution. A change in band shape occurs.

It is shown in the analysis below that changes in salt molality in response to pressure are small and may be neglected. The combined effects of compressing the solution and the macromolecular species are significant and affect the band shape, band position, and buoyant density.

Theory.—Although the effects of pressure in two- and multicomponent sedimentation equilibrium experiments[2-4] have been considered by previous workers, the problem is examined here with special reference to the formation of the density gradient and the behavior of neutrally buoyant macromolecules. For a two-component system at equilibrium in a centrifugal field at constant temperature, the thermodynamic relation,

$$M_2(1 - \bar{v}_{2,P}\,\rho)\omega^2 r\,dr = \left(\frac{\partial \mu_2}{\partial m_2}\right)_P dm_2, \tag{1}$$

is valid.[5] This expression remains valid for free solute[6] in the three-component system at low polymer concentration. In equation (1), M_2 is the molecular weight, $\bar{v}_{2,P}$ the anhydrous partial specific volume, $\mu_2(P, m_2)$ the chemical potential, and m_2 the molality of the solute. The density of the solution, the radial distance, and the angular velocity are $\rho(P, m_2)$, r, and ω, respectively. In equation (1) $\bar{v}_2$, $(\partial\mu_2/\partial m_2)$, and ρ are pressure-dependent variables.

The first two variables are expressed in terms of first-order expansions in pressure about a pressure of 1 atm by Taylor's theorem. Higher-order terms in these expansions are small and therefore neglected. Throughout this paper P will refer to the pressure above atmospheric pressure.

$$\left(\frac{\partial \mu_2}{\partial m_2}\right)_P = \left(\frac{\partial \mu_2}{\partial m_2}\right)_{P=0} + \left(\frac{\partial^2 \mu_2}{\partial P \partial m_2}\right) P.$$

Substituting the relation $(\partial \mu_2/\partial P)_{m_2} = M_2 \bar{v}_2$ into the above equation gives a relation in terms of experimentally accessible variables,

$$\left(\frac{\partial \mu_2}{\partial m_2}\right)_P = \left(\frac{\partial \mu_2}{\partial m_2}\right)_{P=0} + M_2 \left(\frac{\partial \bar{v}_2}{\partial m_2}\right)_{P=0} P. \tag{2}$$

Similarly,

$$\bar{v}_{2,\,P} = \bar{v}_{2,\,P=0} + \left(\frac{\partial \bar{v}_2}{\partial P}\right)_{P=0} P.$$

Introducing the relation for the partial specific isothermal compressibility of the solute, $\kappa_2 \equiv -[1/\bar{v}_2(d\bar{v}_2/dP)]_{P=0,m_2}$

$$\bar{v}_{2,\,P} = \bar{v}_{2,\,P=0}(1 - \kappa_2 P). \tag{3}$$

By a similar Taylor's expansion, the density of the solution may be expressed in terms of the isothermal compressibility coefficient of the solution, κ, at $P = 0$, and the density, $\rho^0(m_2)$,[7] of the solution of molality, m_2, at atmospheric pressure.

$$\rho = \rho^0/(1 - \kappa P) \tag{4}$$

Upon substitution of the effects of pressure, equations (2), (3), and (4), the differential equation (1) becomes

$$\frac{dm_2}{dr} = \frac{M_2\left[1 - \dfrac{(1 - \kappa_2 P)}{(1 - \kappa P)}\,\bar{v}_2{}^0\rho^0\right]\omega^2 r}{\left(\dfrac{\partial \mu_2}{\partial m_2}\right)^0 + M_2\left(\dfrac{\partial \bar{v}_2}{\partial m_2}\right)^0 P}, \tag{5}$$

Equation (5) provides a means of calculating the composition density gradient,

$$\left(\frac{d\rho}{dr}\right)^0 = \frac{dm_2}{dr}\left(\frac{d\rho}{dm_2}\right)^0 = \left(\frac{d\rho}{dm_2}\right)^0 \frac{M_2\left[1 - \dfrac{(1 - \kappa_2 P)}{(1 - \kappa P)}\,\bar{v}_2{}^0\rho^0\right]\omega^2 r}{\left(\dfrac{\partial \mu_2}{\partial m_2}\right)^0 + M_2\left(\dfrac{\partial \bar{v}_2}{\partial m_2}\right)^0 P}, \tag{6}$$

and by comparison with the comparable equation at atmospheric pressure,

$$\left(\frac{d\rho}{dr}\right)^0 = \left(\frac{d\rho}{dm_2}\right)^0 \frac{M_2[1 - \bar{v}_2{}^0\rho^0]\omega^2 r}{(\partial \mu_2/\partial m_2)^0} \equiv \frac{\omega^2 r}{\beta^0}, \tag{7}$$

we can estimate the effect of pressure on salt distribution. Equation (7) defines β^0, a parameter previously calculated by Ifft, Voet, and Vinograd[8] and designated by them as β. Because the pressure correction terms are small, equation (6) may be simplified by expansion, retaining only first-order terms in the corrections. Incorporating the definition of β^0 expressed by equation (7),

$$\left(\frac{d\rho}{dr}\right)^0 = \frac{\omega^2 r}{\beta^0}\left\{1 - \left[\frac{\bar{v}_2{}^0\rho^0(\kappa - \kappa_2)}{1 - \bar{v}_2{}^0\rho^0} + \frac{M_2\left(\frac{\partial\bar{v}_2}{\partial m_2}\right)^0}{\left(\frac{\partial\mu_2}{\partial m_2}\right)^0}\right]P\right\} \tag{8}$$

$$\left(\frac{d\rho}{dr}\right)^0 \equiv \frac{\omega^2 r}{\beta^0}\{1 - \phi P\}.$$

The quantity ϕ has been evaluated for CsCl solutions, Table 1. Pohl's compressibility data,[9] and data for ρ^0 and $\bar{v}_2{}^0$ used in reference 8 were employed in the calculations.[10]

TABLE 1

PRESSURE CORRECTION TERMS FOR THE COMPOSITION DENSITY GRADIENT, CsCl 25°C

ρ^0	κ atm^{-1}, $\times 10^6$	κ_2 atm^{-1}, $\times 10^6$	$\bar{v}_2{}^0\rho^0$	$M_2\left(\frac{\partial\bar{v}_2}{\partial m_2}\right)^0 / \left(\frac{\partial\mu_2}{\partial m_2}\right)^0$ $\times 10^6$	ϕ atm^{-1}, $\times 10^6$
1.3	34.2	−33.4	0.342	42.4	77.5
1.5	29.5	−15.9	0.405	26.8	57.7
1.7	25.8	−5.9	0.464	21.8	49.2

As the pressure at the center of a 1.1 cm CsCl column, $\rho^0 = 1.7$ gm cm^{-3}, at 44,770 rpm is approximately 130 atm,[11] the ϕP correction terms will generally be less than 0.01 and are therefore neglected. The corrections become smaller if shorter CsCl columns are used. The advantage of neglecting the pressure correction to the salt distribution is that ρ^0 may then be calculated with the equation,

$$\rho^0 = \rho_e{}^0 + \int_{r_e}^{r}\left(\frac{d\rho}{dr}\right)^0 dr = \rho_e{}^0 + \int_{r_e}^{r}\frac{\omega^2 r}{\beta^0}\,dr, \tag{9}$$

where r_e is the radius at which the molality of CsCl is that of the initial homogeneous solution, and $\rho_e{}^0$ is the initial density at atmospheric pressure. The isoconcentration distance, r_e, has been determined[8] for different salts as a function of $\rho_e{}^0$ and ω^2. Since the effect of pressure on salt redistribution is small, these data remain valid.

Having established that significant salt redistribution is not caused by the pressures encountered during density gradient analyses, we obtain the *physical density gradient* by differentiating equation (4) with respect to r,

$$\frac{d\rho}{dr} = \frac{1}{1 - \kappa P}\left(\frac{d\rho}{dr}\right)^0 + \frac{\rho^0(m)}{(1 - \kappa P)^2}\frac{d(\kappa P)}{dr}. \tag{10}$$

Introducing $dP/dr = \rho\omega^2 r$, equation (10) for the case of constant compressibility coefficient becomes

$$\frac{d\rho}{dr} = \frac{1}{1 - \kappa P}\left[\left(\frac{d\rho}{dr}\right)^0 + \frac{\kappa\rho^0{}^2\omega^2 r}{(1 - \kappa P)^2}\right]. \tag{11}$$

Neglecting pressure-correction terms of the order of 1% of $d\rho/dr$, the above equation is

$$\frac{d\rho}{dr} = \left[\left(\frac{1}{\beta^0}\right) + \kappa\rho^0{}^2\right]\omega^2 r \equiv \frac{\omega^2 r}{\beta}. \tag{12}$$

The second term in equation (12) is the significant compression term and is tabulated with $1/\beta^0$ in Table 2 for CsCl solutions. The values for the compressibility coefficient κ are interpolated from the data of Pohl.[9] His data are extrapolated linearly from $\rho = 1.4$ to $\rho = 1.8$ gm cm^{-3}.

With the aid of equation (12) it has been demonstrated[12] in the case that $\bar{v}_s$ is a function only of solute activity and pressure that the buoyancy condition in a density gradient experiment is [13]

$$\rho_0{}^0 = \frac{1}{\bar{v}^0{}_{s,0}} \left\{ 1 - \frac{(\kappa - \kappa_s)P_0}{1 - \left(\frac{\partial \rho_0{}^0}{\partial a_1{}^0}\right)_P \left(\frac{da_1{}^0}{d\rho^0}\right)} \right\}. \tag{13}$$

The slope of the $\rho_0{}^0$ versus P_0 relation is $-\psi/\bar{v}^0{}_{s,0}$, where ψ is $(\kappa - \kappa_s)/[1 - (\partial \rho_0{}^0/\partial a_1{}^0)_P (da_1{}^0/d\rho^0)]$. In the following experiments, a study of the dependence of $\rho_0{}^0$ on pressure is made. This dependence is found to be linear.

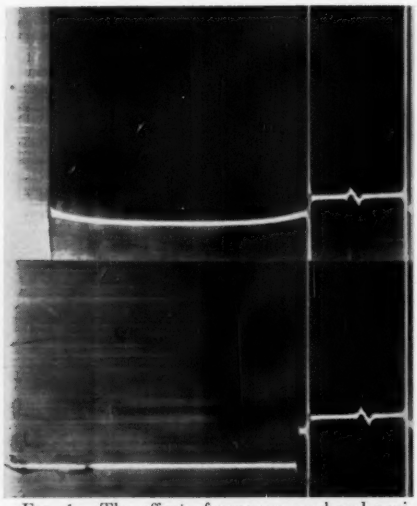

FIG. 1.—The effect of pressure on band position of T-4 DNA in CsCl solution $\rho_c{}^\circ = 1.699$ g. cm.$^{-3}$ at 25°C at 44,770 rpm. The pressure at band center in the lower part of the photograph is 54.5 atm. and in the upper part is 170.7 atm.

The Determination of ψ.—The value of ψ may be measured with adequate accuracy by noting the displacements of equilibrium bands upon changing the pressure. This was accomplished in two ways: An immiscible oil was layered in successive increments on a short column of CsCl solution containing a buoyant macromolecular species and rotated after each increment until equilibrium was attained. The cell containing a band was rotated to equilibrium at varying angular velocities.

Experimental.—Materials: The CsCl was obtained from Maywood Chemical Co., Maywood, N. J., and recrystallized three times. Emission spectroscopic analyses performed in the Department of Geology showed less than 0.03% metallic impurities. The silicone oil was Dow Corning 550 fluid, Lot No. 88–161. According to the manufacturer, it is a methyl- and phenyl-substituted polysiloxane containing less than 10 parts per million of metal salt impurity. The FC-43 fluoro-chemical was obtained from the Minnesota Mining and Manufacturing Co. and is stated to be triperfluorobutylamine. The tobacco mosaic virus strain U 1 (TMV) was kindly supplied by Professor S. Wildman, University of California at Los Angeles. The stock solution contained 5.47 mg/ml TMV in 0.001 versene, pH 7.5. The preparation of the T-4 bacteriophage DNA has been described.[14] All other chemicals used were reagent grade materials.

Procedure.—The effect of pressure on band position: The oil column experiments were performed with both TMV and DNA solutions. The speed-variation experiments were performed only with DNA solutions. The solutions delivered into the standard 4°, 12-mm, Kel-F centerpiece cell assembly had densities, ρ°, of 1.325 and 1.704 g cm^{-3} for TMV and DNA respectively. The first solution contained 46 μg cm^{-3} of TMV and was buffered with 0.01 M tris at pH 7.0. The second solution was buffered with 0.02 M tris at pH 9. The DNA concentration in terms of E^{260}_{1cm} was 0.085. All experiments were begun by filling cells with 0.02 ml of fluorocarbon, 0.18

ml of CsCl solution, and 0.02 ml of silicone oil. Both the fluorocarbon and the silicone oil were included to provide accurate means of recording the top and bottom menisci of the CsCl column. This column was about one-fourth the length attainable in the standard centerpiece.

The solutions were centrifuged at 25.0°C. and photographed with the schlieren optical system after equilibrium had been established. The actual speeds were evaluated from odometer readings made at the beginning and end of the run. These agreed with the nominal values within ±0.02%. In these short columns at 44,770 rpm, the DNA runs required 14 hours and the TMV runs about 90 minutes to reach equilibrium. A typical pair of exposures is shown in Figure 1. The top and bottom CsCl menisci and the silicone oil meniscus were taken to be the centers of the symmetrical meniscus images. These, as well as the counter balance reference edges and the positive and negative peaks, associated with the inflections in the polymer concentration distributions, were

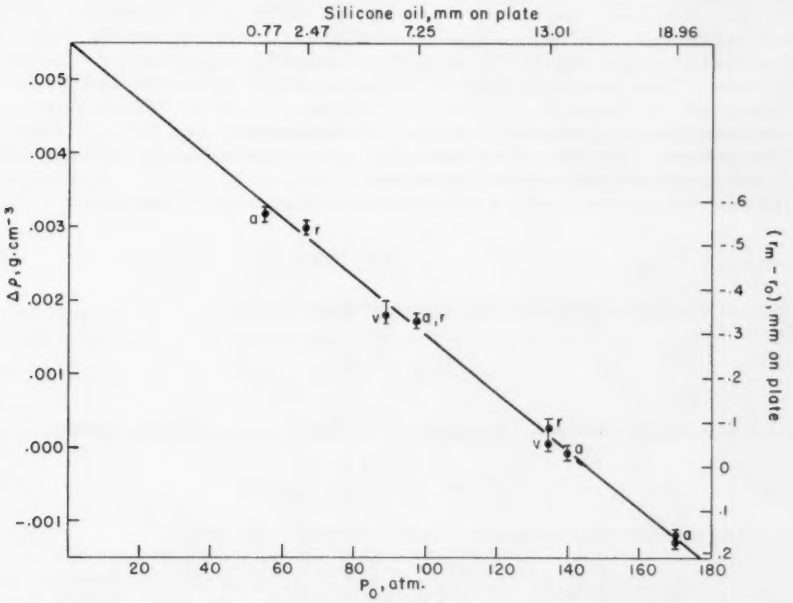

FIG. 2.—Buoyant density increments for T-4 DNA in CsCl solution at various pressures, ρ_c° = 1.699 g. cm.$^{-3}$ at 25°C at 44,770 rpm. Expt. no. 1. *a*, addition of oil; *r*, removal of oil; *v*, points obtained at 31,410 rpm and 39,460 rpm. The coordinates at the top and right-hand side indicate the original data. Slope = -3.94×10^{-5} g. cm.$^{-3}$/atm. The maximum error intervals are indicated.

measured with a coordinate plate and film Comparator Model M 2001-P, Gaertner Scientific Co. Chicago, Illinois. Reading accuracy was ±0.01 mm, which corresponds to ±5 microns in the cell. The band position was taken to be the average of the distances associated with the positive and negative peaks.

In the oil column experiments, the cells were reopened at the filling hole after the first equilibration and 0.16 ml silicone oil added. The solutions were again run to equilibrium. The procedure was repeated twice again. Silicone oil was similarly removed with a 24-gauge needle in two or three stages.

In one of the DNA runs, after the cell was filled with silicone oil and centrifuged to equilibrium, the angular speed was dropped to 31,410 rpm, then raised to 39,460 rpm, and again raised to 44,770 rpm. Thirty-five hours were required for equilibrium at 39,460 rpm and 65 hours at 31,410 rpm. The slow step in these experiments was band motion. The band width became constant after 22 hours at both speeds. After 5 days continuous running, the band returned to its original position at 44,770 rpm as indicated by the double point at 170 atm (Fig. 2).

The density of the silicone oil at 25°C. was measured in a calibrated 0.3 ml micropipet. The density was 1.067 ± 0.001 g cm^{-3}.

The Effect of Pressure on the Composition Density Gradient.—In the theoretical part of this paper, it was noted that the composition density gradient is insensitive to pressure. To check this conclusion, the effect of pressure on the refractive index gradient was measured. The 4° Kel-F, 12-mm standard cell was filled with 0.02 ml of fluorocarbon, 0.34 ml of CsCl solution, $\rho^0 = 1.339$ gm cm^{-3} and 0.02 ml of silicone oil. The rotor was brought to 44,770 rpm as rapidly as possible, and schlieren images at a bar angle of 55° were photographed immediately upon reaching this speed and again after 10 hours, at equilibrium. The experiment was repeated after thorough homogenizing of the CsCl solution and the addition of 0.34 ml of silicone oil. The composition density gradient was obtained at r_e from the difference in elevations between the early and equilibrium exposures.

For these experiments, tracings were made of 20-fold cell to vellum enlargements with an Omega D-2 enlarger. The CsCl meniscus was used for lateral orientation and the image of a horizontal wire mounted just in front of the schlieren camera for vertical orientation. Elevations proportional to the compression density gradient were measured with an accuracy of 0.4 per cent.

Calculations.—For evaluation of the results in accordance with equation (13), P_0, the pressure at band center, and ρ_0^0, the buoyant density at atmospheric pressure, must be derived from the experimental data. The pressure is the sum of the pressures generated by the action of the field in the silicone oil and in CsCl solution over the band.

The pressure at any point in a liquid volume is obtained by integration of the equation

$$\frac{dP}{dr} = \rho\omega^2 r. \tag{14}$$

In order to integrate this expression, the r dependence of ρ is needed.

In a solution,

$$\rho = \rho_\alpha + \int_{r_\alpha}^{r} \frac{\omega^2 r}{\beta}\, dr, \tag{15}$$

where r_α is an arbitrary reference. It is sufficiently accurate to assume that β is a constant.

$$\rho = \rho_\alpha + \frac{\omega^2}{\beta}\left(\frac{r^2 - r_\alpha{}^2}{2}\right) \tag{16}$$

Substituting this expression into equation (1) and integrating from r_1 to r_2,

$$P_2 - P_1 = \rho_\alpha\omega^2\left(\frac{r_2{}^2 - r_1{}^2}{2}\right) + \frac{\omega^4}{2\beta}\left[\frac{(r_2{}^4 - r_1{}^4)}{4} - r_\alpha{}^2\,\frac{(r_2{}^2 - r_1{}^2)}{2}\right]. \tag{17}$$

To simplify computation, r_α is so selected that the term in the brackets in equation (17) vanishes. For this condition, r_α is the root mean square between r_1 and r_2. In all cases the use of the arithmetic mean for r_α did not introduce a significant error.

$$r_\alpha = \sqrt{\frac{r_2{}^2 + r_1{}^2}{2}} = \frac{r_2 + r_1}{2} + \frac{1}{8}\,\frac{(r_2 - r_1)^2}{r_2} \cdots$$

The pressure at band center caused by the CsCl solution was evaluated as follows: The quantity r_α is the root mean square radius between band center, r_0, and the CsCl meniscus, r_{cm}. The density ρ_α is obtained with sufficient accuracy with equation (9), assuming β° constant, the limits of integration being r_e and r_α. This neglects the effect of pressure on ρ_α. Substitution into equation (5) yields

$$P_{r_0,\,CsCl} = \frac{\rho_\alpha\omega^2}{1.013 \times 10^6}\,\frac{(r_0{}^2 - r_{cm}{}^2)}{2} \tag{18}$$

The numerical coefficient is introduced in order to express pressure in atmospheres. ρ_α was found to vary only 0.12% in a given series over the entire range of oil column lengths and was therefore considered constant.

In the oil column treated as a one-component system, $1/\beta^0 = 0$, and $1/\beta = \kappa'\rho^{02}$, equation (12), where κ' is the isothermal compressibility of the oil. The same arguments used for the CsCl column apply here. In this case, however, the compressibility and the pressure contribution of the oil are larger; the effect of pressure on ρ_α is therefore taken into account. For a good estimate of the pressure P_α, equation (14) is integrated between the oil meniscus, r_{om}, and r_α, under the assumption that the density is constant.

$$P_\alpha = \frac{\rho^0\omega^2}{1.013 \times 10^6}\frac{(r_\alpha^2 - r_{om}^2)}{2} = \frac{\rho^0\omega^2}{1.013 \times 10^6}\frac{(r_{cm}^2 - r_{om}^2)}{4} \tag{19}$$

The r_α was eliminated with the definition of r_α. Upon substituting $\rho_\alpha = \rho^0(1 + \kappa'P_\alpha)$ and equation (19) into equation (17), and letting r_2 be the cesium chloride meniscus, r_{cm}, and r_1 be the oil meniscus, r_{om},

$$P_{cm} = \rho^0\left\{1 + \kappa'\left[\frac{\rho^0\omega^2}{1.013 \times 10^6}\frac{(r_{cm}^2 - r_{om}^2)}{4}\right]\right\}\left(\frac{(r_{cm}^2 - r_{om}^2)}{2}\right). \tag{20}$$

The pressure at band center is the sum of the pressures given by equations (18) and (20).

Now the shifts in band position must be expressed in density units. The buoyant density at atmospheric pressure, ρ_0^0, is calculated with equation (9). In order to express the dependence of β^0 on r, β^0 is expanded:

$$\beta^0 = \beta_\gamma{}^0 + \left(\frac{d\beta^0}{dr}\right)_\gamma (r - r_\gamma)$$

where r_γ is again an arbitrary reference radius. Integrating and evaluating r_γ so that the $(d\beta^0/dr)_\gamma$ term equals zero as before, the following equations are obtained,

$$r_\gamma = \frac{r_2 + r_1}{2} + \frac{(r_2 - r_1)^2}{12\, r_2} \cdots$$

and

$$\rho_2{}^0 - \rho_1{}^0 = \frac{\omega^2}{\beta_\gamma{}^0}\left(\frac{r_2^2 - r_1^2}{2}\right).$$

As in the previous case, the selection of β^0 at the arithmetic mean rather than at $r\gamma$ will not introduce a significant error.

Since the density shifts due to the pressure variation are very small, the quantity $\Delta\rho = \rho_0{}^0 - \rho_e{}^0$ was evaluated and not $\rho_0{}^0$ itself. The desired expression is

$$\Delta\rho = \rho_0{}^0 - \rho_e{}^0 = \frac{\omega^2}{\beta_e{}^0}\left(\frac{r_0^2 - r_e^2}{2}\right). \tag{21}$$

The following relation utilizing the original comparator data (capital R's) and the magnification factor, MF, was used,

$$(r_0^2 - r_e^2) = \frac{2 \times 5.725}{MF}\left(R_0 - \frac{R_{cm} + R_{fm}}{2}\right) + \frac{1}{(MF)^2}\left(R_0^2 - \frac{R_{cm}^2 + R_{fm}^2}{2}\right), \tag{22}$$

where R_{fm} is the radius of the fluorocarbon meniscus.

According to theory, a plot of $\Delta\rho$ versus P_0 should yield a straight line of slope $-\psi/\bar{v}^0{}_{s,0}$ and intercept $(1/\bar{v}^0{}_{s,0} - \rho_e{}^0)$. Figures 2 and 3 present the data obtained for one series each of DNA and TMV. The value of $\bar{v}^0{}_{s,0}$ was determined from the intercept. From the intercept and the slope, values for ψ were obtained. Duplicate experiments in each case were performed.

In the measurements, the radii were located with a precision of 0.01 mm on the plate. The values of P_0 were calculated with no significant error. The values of $\Delta\rho$ are very sensitive to the accuracy of measurement because these values are derived from the difference of the squares of two numbers of comparable magnitudes. The maximum error in each of the $\Delta\rho$ values was

calculated to be ± 0.00012 gm cm^{-3}. The two points obtained at lower speeds show an asymmetric maximum-error interval. This is due to the difficulty in measuring the position of the broader bands at lower speeds. The flare at each maximum had to be used to find the peak. It was known from observations of the relation of the flare to the actual maximum on photographs at 44,770 rpm that the flare from both peaks is displaced approximately 0.02 mm toward the center of rotation. This corresponds to an error of -0.00010 gm cm^{-3}.

The probable error of the function $\Delta\rho$ was computed using an expression given in Margenau and Murphy.[15] Assuming a probable error of 0.005 mm in each comparator measurement, a probable error for the function, $\Delta\rho$, was found to be 0.00003 gm cm^{-3}.

The adherence of the points after the oil removal to the straight line showed that no detectable evaporation occurred. The points in general can be determined with an accuracy of 0.0001 gm cm^{-3}. This corresponds to an evaporative loss of 0.026 mg of H_2O and a meniscus shift of 0.5μ. Because the meniscus can be located with an accuracy of only 10 μ, it is clear that the band position is a much more sensitive test for evaporation than the change in meniscus height. A density shift of 0.004 gm cm^{-3} is required to cause an observable shift in CsCl column length.

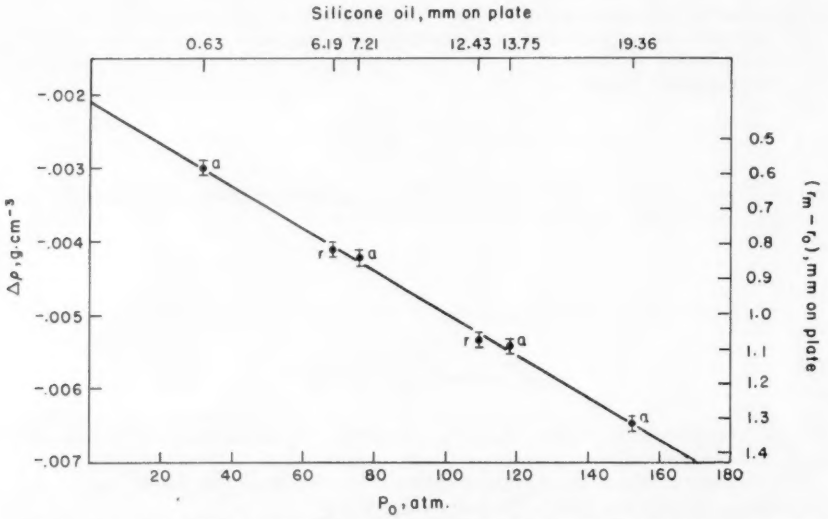

FIG. 3.—Buoyant density increments for TMV in CsCl solution at various pressures, ρ_r° = 1.324 g. cm.$^{-3}$ at 25°C at 44,770 rpm. Expt. no. 3. *a*, addition of oil; *r*, removal of oil. The coordinates at the top and right-hand side indicate the original data. Slope = -2.88×10^{-5} g. cm.$^{-3}$/atm The maximum error intervals are indicated.

Results and Discussion.—The examination of the effects of pressure-dependent terms in equilibrium sedimentation of macromolecules in a density gradient illuminates two important interrelated problems, solvation and compression. These effects are also encountered in conventional sedimentation equilibrium but are smaller and frequently neglected.

The effects of compression on the physical density gradient in the CsCl solutions have been analyzed. The result, Table 2, is to increase the physical density gradient by 8.5–10.8%. These effects are independent of speed. At a density of 1.7 gm cm^{-3}, the compression gradient is 9% of the composition gradient.

That the compression gradient may be simply added to the composition gradient is shown in experiments in which the refractive index gradient at equilibrium was

TABLE 2

THE EFFECT OF COMPRESSION ON THE PHYSICAL DENSITY GRADIENT IN CsCl SOLUTIONS AT EQUILIBRIUM IN THE ULTRACENTRIFUGE AT 25°C

ρ^0	$1/\beta^0 \times 10^{10}$	$\kappa\rho^{02} \times 10^{10}$	$\frac{1/\beta^0 + \kappa\rho^{02}}{1/\beta^0} = \frac{1/\beta}{1/\beta^0}$
1.2	5.042	0.543	1.108
1.3	6.468	0.586	1.091
1.4	7.429	0.629	1.085
1.5	8.034	0.673	1.084
1.6	8.351	0.714	1.085
1.7	8.400	0.757	1.090
1.8	8.231	0.797	1.097

evaluated in a short CsCl column with and without a layer of silicone oil. The effect on the net refractive index gradient at the root mean square position in the cell due to this layer of oil, which corresponded to a pressure increment of 72.1 atmospheres, was -1.3%. This result is within the experimental measuring errors and the effect predicted by the theory, Table 1. It is consistent with the previously obtained agreement[8] between the composition density gradient obtained optically at the pressure in the cell and calculated from physical chemical data obtained at atmospheric pressure. Three approaches now show that the CsCl–water distribution is not significantly changed by compression, even though the solution is compressed and the physical density gradient increased.

In density gradient experiments the macromolecular species does not respond only to the physical density gradient. Of importance is the *effective density gradient*, which is shown in the preceding paper[12] to be

$$\left(\frac{d\rho}{dr}\right)_{\text{eff}} = \left[\frac{1}{\beta^0} + \psi\rho_0{}^{02}\right](1 - \alpha)\omega^2 r \tag{23}$$

where

$$\alpha = \left(\frac{\partial\rho_0{}^0}{\partial a_1{}^0}\right)_P \left(\frac{da_1{}^0}{d\rho^0}\right).$$

The quantity ψ must therefore be known in order to evaluate correctly the effective density gradient and the molecular weight of either the anhydrous or the hydrated species.

The values of ψ and $\bar{v}^0_{s,0}$ obtained from the oil column experiments are given in Table 3. The numbers in the fifth column, $1/\bar{v}^0_{s,0}$ are the values of the composition variable $\rho_0{}^0$ when the band is at atmospheric pressure. The values listed as $\rho_0{}^0$ are values calculated for the pressure at the middle of a 1.1 cm column of CsCl solution at 44,770 rpm. Thus significant deviations in $\rho_0{}^0$ will be encountered by workers banding the same material at different radii in the cell.

If proper experimental data are available, ψ can be used to calculate the apparent compressibility of the solvated polymer. A more complete discussion of this matter

TABLE 3

EFFECT OF PRESSURE ON BUOYANT MOLECULES IN A DENSITY GRADIENT

Material	Expt. No.	$\psi \times 10^6$ atm^{-1}	$\frac{1}{\bar{v}^0_{s,0}}$	$\frac{1}{\bar{v}^0_{s,0}}$	ρ^0
T-4 Bacteriophage	1	23.1	0.587	1.704	1.699
DNA	2	23.5	0.587	1.703	1.698
Tobacco mosaic	3	21.8	0.756	1.322	1.319
virus, strain U 1	4	21.5	0.757	1.321	1.318

is presented in the preceding article.[16] If the effects of solvation are neglected, the value of κ_S for *TMV* obtained from ψ and from Pohl's compressibility data is 12.9×10^{-6} atm^{-1}. This value agrees favorably with the value 10×10^{-6} atm^{-1} which Jacobson[17] obtained for various proteins in dilute salt solutions. The agreement may be the result of coincidence, because the effects of solvation have been neglected.

For *T-4 DNA* ψ was found to be 23.3×10^{-6} atm^{-1}. The parameter α for *DNA* in CsCl is 0.24.[16] These numbers may now be used to calculate the difference in compressibility, $\kappa - \kappa_s = 17.7 \times 10^{-6}$ atm^{-1}. From this difference κ_s is found to equal 8.7×10^{-6} atm^{-1}. It should be stressed that κ_s is an apparent compressibility. It not only includes the compression of the solvated species, but also a pressure dependence of the amount of hydrated water.

The use of an insoluble marker material, such as a thin film of plastic, has been suggested by Szybalski.[18] Solvation effects for such a material should be negligible

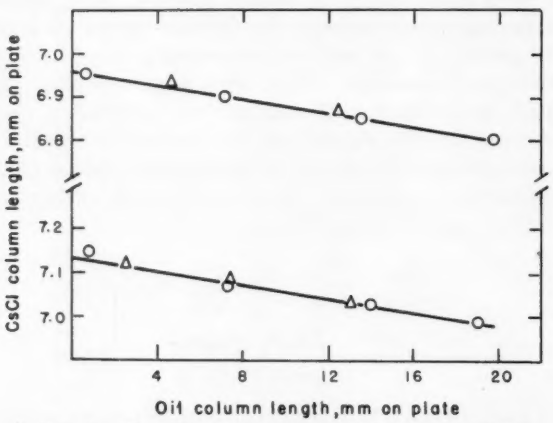

FIG. 4.—The effect of pressure on CsCl solution column length. ○, addition of oil; △, removal of oil. The lower line is from expt. no. 1 and the upper line is from expt. no. 2.

because surface areas are relatively small. Large pressure effects on marker positions are to be expected and have been observed.[18]

DNA samples, denatured, isotopically substituted, or of differing composition, can be used as density markers, providing that the dependence of ψ and α on pressure for the marker is the same as the sample under investigation. The effective density gradient, equation (23), is used to calculate density differences in such experiments.

Cell Distortion.—The CsCl column length was found to change in a characteristic manner with the level of the supernatant silicone oil (Fig. 4). The two sets of points correspond to two independent experiments. In these, the cell was disassembled and reassembled with a solution of slightly different density. The linear and elastic response reflects the behavior of a cell assembly with a Kel-F centerpiece and quartz windows. The observed decrease in CsCl column length of 2.1% is 10.8 times larger than that attributable to the compression of the solution.

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‡ U.S. Public Health Service Research Fellow of the Division of General Medical Sciences.

** Contribution No. 2701.

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$$\kappa_2 = \kappa + \frac{1}{\rho^\circ \bar{v}_2^\circ}(\kappa_i - \kappa)$$

The quantity κ_i is the intercept at $Z_2 = 1$ of the tangent to the κ versus weight fraction of CsCl' Z_2, curve.

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AMBIVALENT rII MUTANTS OF PHAGE T4

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The *r*II mutants of phage T4 differ from the standard type in being inactive on strains of *Escherichia coli* lysogenic for phage λ. While the mutants attach to the cells and inject their DNA, progeny fail to appear. In this paper it is shown that one or another subset of the *r*II mutants can become active as a result of modification of the host, either by mutation or by the action of 5-fluorouracil. The effects are characterized by extreme specificity, applying only to certain *r*II mutations at certain points within the two *r*II cistrons. The phenomenon resembles what has been called, in many other systems, "allele-specific suppression,"[1] in which a muta-

tion outside a genetic region controlling a particular function suppresses the effects of some of the mutations within the region.

An advantage of the phage system is that a given genetic message, in the form of a phage mutant, can be readily inserted into different hosts. The stimulus for the present investigation derives from the possibility that modifications of the host affecting the expression of specific phage mutations might be a key to the detection of alterations in the components of the cellular system for translating genetic information.

Materials and Methods.—Bacterial strains: For crosses and for non-selective plating *E. coli* B was used. *E. coli* KB is the strain previously designated[2] as K. It was derived from K12(λ), which was cured of lysogenicity to produce the sensitive strain K12S (Lederberg and Lederberg[3]), and transmitted through Luria to Benzer, who relysogenized it with phage λ obtained from K12(λ). *E. coli* KT is otherwise known as 112–12 (λh #3). It was derived from K12(λ) by treatment with ultraviolet light to produce a cured strain 112-12 (Wollman[4]), was later lysogenized with a mutant of λ and found its way through Epstein to Tessman to us.

Designation of rII mutants:[5] All were derived from phage T4B, with the exception of those designated by ED, which are spontaneous mutants derived from T4D. *Spontaneous mutants* have either no prefix, or the prefixes SN or SD. A single letter prefix from A through J designates spontaneous mutants derived from revertants of spontaneous *r*II mutants. *Mutagen-induced mutants* are prefixed as follows: NA, NB or NT induced by nitrous acid; EM by ethyl methane sulfonate; HB by hydroxylamine; N or M by 5-bromouracil or 5-bromodeoxyuridine; AP by 2-aminopurine; DAP by 2,6-diaminopurine; BC by 5-bromodeoxycytidine; P by proflavine; PT, PB by heat at low pH; UV by ultraviolet light. Many of the mutants were contributed by J. Drake, R. Edgar, E. Freese, M. Meselson and I. Tessman.

Genetic mapping of rII mutants was done by techniques previously described.[5] Strain KB was used as the selective indicator except when crosses involved *r*II mutants that were too active on KB, in which case strain KT was employed. For conventional crosses, as used in the analysis of *r*EM64, the procedure of Chase and Doermann[6] was followed.

Media: Unless otherwise noted, the medium was broth (1% Difco bacto-tryptone plus 0.5% NaCl). For plates, 1.2% agar was added for the bottom layer and 0.7% for the top layer. In experiments involving 5-fluorouracil, a synthetic medium (M9S) was used containing, per liter of solution, 5.8 gm Na_2HPO_4, 3.0 gm KH_2PO_4, 0.5 gm NaCl, 1.0 gm NH_4Cl, 0.25 gm $Mg\,SO_4 \cdot 7H_2O$, 2.7 mg $FeCl_3 \cdot 6H_2O$, 4.0 gm glucose, 20 mg L-tryptophan, and 2.5 gm Difco vitamin-free casamino acids. M9 buffer is the same medium minus the carbon compounds. *5-fluorouracil* was kindly donated by the Hoffman-LaRoche Company.

Burst size measurements were made in broth at 37°C using exponentially growing cultures at a cell concentration of 2 to 3 × 10^8/ml. Phage was added in the presence of 5 × 10^{-3} *M* NaCN at a multiplicity of less than 0.01 phage per bacterium. After allowing 15 min for adsorption, a sample was diluted 10^4-fold. (If a measurement required the elimination of unadsorbed phage, T4 antiserum was added to the adsorption tube 10 min after infection.) After 60 min of growth the cells were lysed with chloroform and the phage yields were assayed on *E. coli* B. The number of cells infected by *r*II mutants cannot be readily assayed, but, since adsorption under the conditions used was virtually complete, the burst size was calculated using the titer of the phage stock.

Treatment of bacteria with ethyl methane sulfonate (EMS): Induction of mutations in the bacterial strains was done according to a method communicated by E. Lin. One drop of EMS (Distillation Products Industries) was dissolved in 5 ml of an overnight aerated culture of bacteria in broth. After 2 hours' incubation at 37°C, a tenfold dilution was made into fresh broth and aerated overnight. This culture was treated with EMS, the cycle being repeated a total of six times. To measure the effectiveness of the treatment, the proportion of bacteria resistant to phage T6 was measured. This proportion increased more than 100-fold in the treated series and reached a maximum by the third cycle, but it remained almost unchanged in controls unexposed to EMS.

Results.—Ambivalent rII mutants: *r*II mutations have been located in a small portion of the genetic map of the phage T4, at more than three hundred distinct

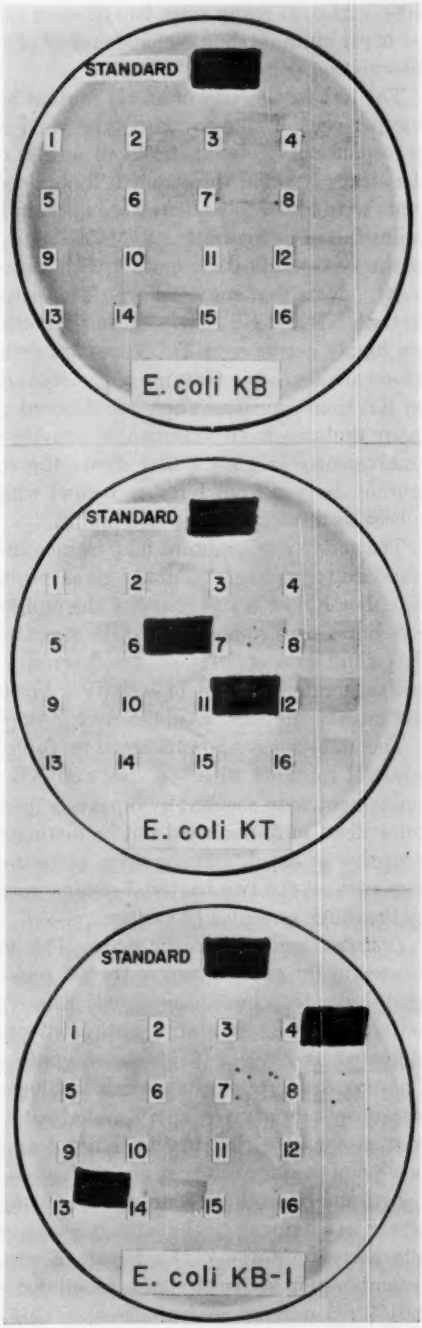


FIG. 1.—Spot tests for activity of various *r*II mutants on three bacterial strains. Each plate was seeded with about 10^8 bacteria in a top layer of soft agar. Streaks of standard type T4 and sixteen different *r*II mutants were added (to the right of each number), by means of a sterile paper strip dipped into a stock of the mutant phage, and the plates were incubated overnight at 37°C.

Standard type (r^+) is active on all three bacterial strains. Ambivalent mutants of subset 1 are illustrated by spots 6 and 11. Spots 4 and 13 are ambivalent mutants of subset 3. The occasional plaques are due to revertants present in the mutant stocks. The mutants are:

(1) *r*131,
(2) *r*1272,
(3) *r*289,
(4) *r*AP129,
(5) *r*859,
(6) *r*NT332,
(7) *r*744,
(8) *r*607,
(9) *r*NT341,
(10) *r*BC24
(11) *r*C204,
(12) *r*1011,
(13) *r*N38,
(14) *r*UV272,
(15) *r*N84, and
(16) *r*P58.

sites. Also, at many sites, two or more kinds of mutations are known. This large set of *r*II mutants should include many of the various possible changes in the genetic information elements.

The test for activity of an *r*II mutant is a sensitive one, since a small fraction of one per cent of the standard type yield can be readily detected. The difference in response of bacterial strains to certain *r*II mutants was first discovered between the strain KB and the strain KT obtained from Irwin Tessman. Particular mutants were found to be defective on strain KB but to be active on strain KT. This is illustrated in Figure 1. Each of the test plates shown was seeded with bacteria of the strain indicated, spotted with various phage mutants, and incubated overnight. Note that standard type T4 is active (lyses the bacteria producing clearing) on both KB and KT. Of sixteen *r*II mutants inactive on KB, two (spots 6 and 11) are highly active on KT. These are defined as ambivalent mutants of subset 1. There are also some mutants of the converse type (not shown) that are more active on KB than on KT. These are denoted as ambivalent subset two. (In addition, many mutants have considerable activity on both KB and KT but are not clearly ambivalent.) Figure 1 also shows the response of the bacterial strain KB-1, a mutant derived from KB (see below) which serves to identify a third ambivalent subset, as illustrated by spots 4 and 13.

The activity of a mutant may be measured by its burst size as compared with the standard type phage. Table 1 gives results for various *r*II mutants when adsorbed on either KB or KT. Many of the ambivalent mutants of the class active on KT give burst sizes comparable to the standard type. This does not necessarily mean that a full level of "*r*II$^+$ enzyme" activity is produced, since the burst size may be an exaggerated measure of activity. For the class of ambivalent mutants more active on KB, the contrast in behavior of the two bacterial strains is less pronounced.

The ambivalent mutants listed in Table 1 were found by screening several thousand *r*II mutants with spot tests on KB and KT. Those which appeared to be ambivalent were checked by burst size measurements. In cases where two or more ambivalent mutants could not be distinguished by recombination, only one representative is listed. There may, of course, be other ambivalent mutants whose responses on the two bacterial strains were not sufficiently different to be detected by the rough screening procedure.

Distribution in the genetic map: The ambivalent mutants listed in Table 1 are located in the genetic map of the *r*II region at the sites shown in Figure 2. A triangle indicates a mutant more active on KT and a square indicates one with greater activity on KB. Mutants with relatively low activity on both KB and KT are indicated as circles. (The stars denote a third ambivalent subset—see below.) The proportion of sites at which ambivalence has been noted is about 1 in 23 for the subset more active on KT and about 1 in 39 for the reciprocal type. It can be seen at a glance that neither kind of ambivalence is restricted to any particular portion of the map or to one cistron.

In some cases, both ambivalent and non-ambivalent mutations have been observed at what may be the same genetic site. Table 2 lists the data for such possibly alternative mutations. Each pair of mutants, when crossed, gave no observable recombination above the background due to spontaneous reversion (less than about 0.01% recombination in all cases). This does not necessarily prove that the sites

TABLE 1

BURST SIZES OF VARIOUS rII MUTANTS ON *E. coli* STRAINS KB AND KT

	Bacterial Host	
	E. coli KB	*E. coli* KT
Standard type phage		
r^+	100.	100.
Non-ambivalent rII mutants		
r131	0.00	0.00
r1272	0.00	0.03
Ambivalent rII mutants of subset 1		
rHB118	0.00	98.
rHB122	0.00	42.
rC204	0.06	104.
rN11	0.00	127.
rEM64	0.00	99.
rHB32	0.00	19.
rHB35	0.00	100.
rHB80	0.00	6.3
rHB129	0.00	102.
rHB84	0.00	106.
r2074	0.00	20.
rEM84	0.00	79.
rHB74	0.00	72.
rNT332	0.00	72.
rB94	0.00	81.
rHB232	0.00	108.
Ambivalent rII mutants of subset 2		
rSN86	37.	2.8
rHB309	40.	3.0
r154	41.	3.8
rNB3830	5.0	0.3
rN40	10.	0.03
rHB33	16.	1.1
rN89	7.2	0.6
rHB8	50.	4.0
rDAP35	13.	1.1

Burst sizes are expressed as per cent of the burst size of standard type, which was several hundred on each host strain. A zero in the last decimal place indicates a value of less than one for that digit. The values (particularly low ones) are subject to fluctuations from one experiment to another and some are quite sensitive to temperature. Differences less than a factor of two are probably not significant.

are identical. It shows clearly, however, that ambivalence is not merely a property of certain small regions of the map.

A feature not shown in Figure 2 is the number of apparent recurrences at each site. At some of the sites many independent mutations have been observed which may or may not all be ambivalent. For example, four other mutations occurred at the site rHB129 and all were ambivalent in the same way as rHB129. At other sites, ambivalence provides a further criterion (in addition to degree of activity, reversion rate, and response to mutagens) for discriminating between alternative mutations at the same apparent site.

Back cross test of an ambivalent mutant: Since ambivalent mutants are in the minority, one possibility which must be considered is that they owe their properties to additional mutations (outside of the rII region) which affect the behavior of the phage on different bacterial strains. For example, a given rII mutant might have the potential to be fairly active on both strains KB and KT but might happen to harbor a second mutation, having no relation to rII, which prevents the adsorption of the phage to one of them. This would make the mutant appear to be ambivalent. While a mutation affecting adsorption could, of course, be detected directly, other more elusive mechanisms might be imagined. From the fact that at some sites several (and in some cases all) recurrences are ambivalent, it would seem most un-

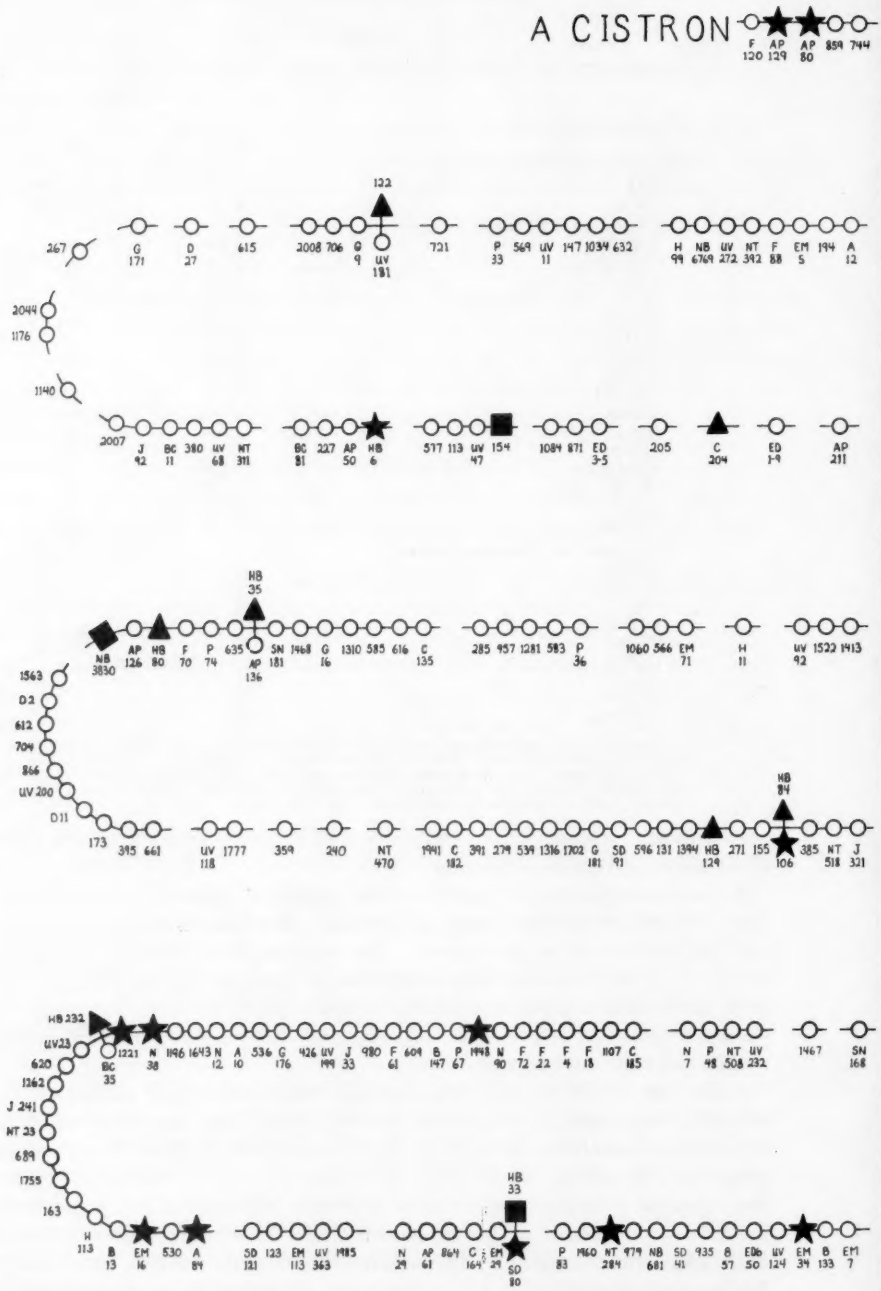
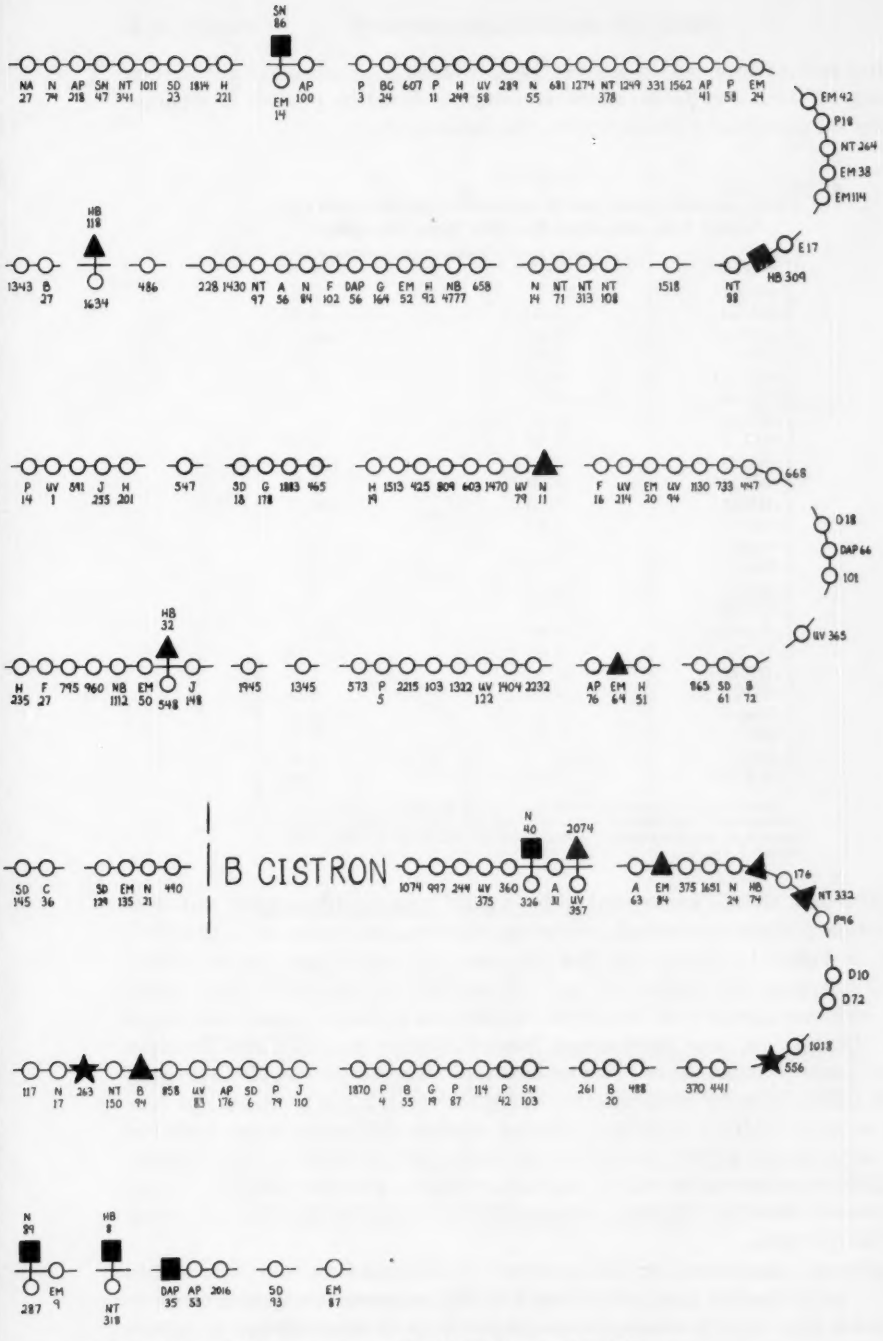


FIG. 2.—Genetic map of the *r*II region, showing the locations of sites at which ambivalent mutants have been found. The breaks in the map indicate segments as defined by the ends of deletions.[6] The order of the segments has been determined as shown. The order of mutations within any one segment has not been determined, but all give recombination with one another.



▲ Ambivalent of subset 1, more active on *E. coli* KT than on *E. coli* KB.
■ Ambivalent of subset 2, more active on *E. coli* KB than on *E. coli* KT.
★ Ambivalent of subset 3, more active on *E. coli* KB-1 than on *E. coli* KB.
○ No pronounced ambivalence noted.

likely that ambivalence can, in general, be attributed to independent extraneous mutations. If such a mutation exists, however, it should be possible to separate it from the *r*II mutation by a back cross to the standard type.

TABLE 2

PAIRS OF AMBIVALENT AND NON-AMBIVALENT MUTANTS FOR WHICH RECOMBINATION HAS NOT BEEN DETECTED

*r*II mutant	Bacterial Host: *E. coli* KB	Bacterial Host: *E. coli* KT
*r*SN86	37.	2.8
*r*EM14	0.00	0.05
*r*HB118	0.00	98.
*r*1634	0.00	0.4
*r*HB122	0.00	42.
*r*UV181	0.04	0.08
*r*HB32	0.00	19.
*r*548	0.00	0.04
*r*HB35	0.00	100.
*r*AP136	0.00	0.00
*r*HB84	0.00	106.
*r*106	0.4	0.1
*r*N40	10.	0.03
*r*326	0.00	0.00
*r*2074	0.00	20.
*r*UV357	0.00	0.00
*r*HB232	0.00	108.
*r*BC35	0.00	0.00
*r*HB33	16.	1.1
*r*SD80	0.00	0.00
*r*N89	7.2	0.6
*r*287	0.02	0.04
*r*HB8	50.	4.0
*r*NT318	0.00	0.04

The mutants of each bracketed pair cannot be distinguished by recombination, but behave differently on the two bacterial strains. Burst sizes are given as per cent of the burst size of the standard type phage.

With this aim, *r*EM64 was crossed with standard type and the progeny plated on B; 348 *r*-type plaques were picked, purified by replating, and tested on KB and KT, as shown in Figure 1. All but one had the same ambivalent character as *r*EM64. This one exception was inactive on both KB and KT and proved to be a double mutant with one mutation at the site of *r*EM64 plus a second mutation in the B cistron. It therefore must have arisen from *r*EM64 by mutation and does not represent a loss of ambivalence by recombination. The proportion of *r*II progeny that lost ambivalence by recombination was thus less than 0.3 per cent. In contrast, a cross of *r*EM64 with *r*C41, a mutant near the beginning of the A cistron (at the same site as *r*AP80), yielded 1.5 per cent r^+ recombinants. The distance from *r*EM64 to either end of the *r*II region is at least as great as to *r*C41. Therefore, the results show that the ambivalence of *r*EM64 cannot be due to any mutation outside the *r*II region.

The effect of 5-fluorouracil on rII mutants: Naono and Gros and their collaborators[7–9] have reported that 5-fluorouracil (5FU), a pyrimidine analog which is incorporated into RNA,[10] causes changes in the rates of incorporation of certain amino acids as well as modification of the properties of β-galactosidase and alkaline phosphatase of *E. coli*. This suggests that the analog may cause specific errors in

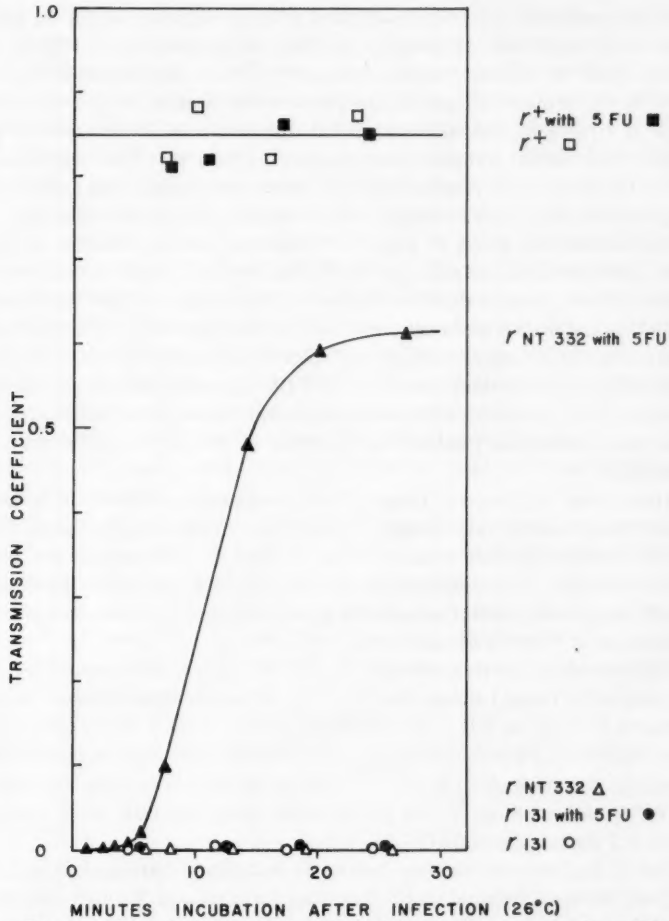


FIG. 3.—Effect of 5-fluorouracil on the activity of *r*II mutants on E. coli KB. The transmission coefficient is the fraction of infected cells yielding at least one progeny particle.

In this experiment a culture of KB growing exponentially in M9S medium was used at a cell concentration of 3.7×10^8/ml. Phage was added in the presence of 0.01 *M* NaCN at a multiplicity of 0.3 per bacterium. After allowing 10 min for adsorption at 37°C anti-T4 serum was added, and 5 min later a sample was diluted 100-fold in *M*9 buffer, then 20-fold at 26°C into M9S + 20 γ/ml thymidine with and without 10 γ/ml 5FU. This is time zero on the graph. At various times thereafter, samples were plated on *E. coli* B using broth plates (this arrests the action of 5FU) to determine the fraction of cells producing at least one progeny particle. The latent period under these conditions was 100 min for the standard type phage in the absence of 5FU.

the translation of the genetic information of the cell. If this idea is correct, certain mutants (having the proper genetic errors) should be stimulated by 5FU.

Many *r*II mutants have been tested for their response to 5FU and some have been found to respond strikingly. Figure 3 shows the effect of 5FU on a responsive and a non-responsive mutant. A convenient index to the activity of a mutant is the

"transmission coefficient," i.e. the fraction of infected cells (strain KB in this case) giving rise to at least one *r*II progeny particle, as assayed by plating on *E. coli* strain B, on which *r*II mutants produce plaques. In this experiment KB cells were infected with the mutant phage (in the presence of cyanide to prevent development) and at time zero, diluted into growth medium with or without 5FU. At various times thereafter, samples were plated on strain B. The transmission coefficient for the responsive mutant, *r*NT332, increased rapidly during the first 20 min of exposure to 5FU. By contrast, *r*131 showed no detectable response.

Under the conditions given in Figure 3, uracil, at a concentration of 20 γ/ml, completely prevented the stimulation of *r*NT332 by 5FU while thymidine, at the same concentration, had no detectable effect. This suggests that the stimulating effect of 5FU is not due to an interference with DNA synthesis. The effect of 5FU on *r*NT332 is a physiological one and not due to mutagenesis, since the progeny phage yielded by KB (in the presence of 5FU) are no more active (in the absence of 5FU) than their parents. 5-Bromouracil at the same molar concentration and under the same conditions produced no increase in the activity of either of these mutants on KB.

5FU causes some inhibition of phage growth in general, as shown by a decreased burst size of the standard type phage. (This is not evident in the figure, since the transmission coefficient still remains close to unity.) However, the increased activity observed for the responsive mutant is over and above this general inhibition. Both the general inhibition and the specific stimulation are insensitive to the concentration of 5FU above about 1γ/ml.

Table 3 shows data for the response to 5FU of various mutants tested on KB. A comparison with Table 1 shows that 12 of the 16 ambivalent mutants of subset 1 are responsive to 5FU on KB. By sharp contrast, a survey of mutants (not belonging to subset 1) chosen arbitrarily from 55 different sites revealed only one (*r*N24) which was responsive to 5FU. Difficulties arise in testing the ambivalent mutants of the subset more active on KB since they generally have considerable activity on KT even without 5FU.

Alteration of the bacterial response pattern by mutation: Strains KB and KT are derived from the same original strain, but they have passed through various treatments in different hands and have had opportunities to accumulate changes. Each has at one time been "cured" of its prophage and re-lysogenized with another strain of λ. Since the presence of prophage λ is necessary for the block against *r*II mutants it was important to determine whether the response pattern is related to the prophage specificity.

This was tested by transferring the prophage from one strain into the other. A culture of KT was induced with ultraviolet light and the resulting phage was used to lysogenize strain K12S, the same non-lysogenic strain that had been used to manufacture the lysogenic strain KB. Two independently-arising lysogenic strains were tested with five ambivalent mutants of subset 1 and five of subset 2 and gave results typical of strain KB. Thus, the identity of the prophage *per se* does not determine the reponse pattern.

The response pattern of the bacteria can, however, be altered by mutation. Such mutants have been isolated from cultures subjected to treatment by ethyl methane sulfonate (see *Methods*). Two kinds of changes were looked for: (1) acquisition

of ability to support the development of an inactive *r*II mutant, and (2) loss of ability to permit development of an active *r*II mutant. Both types can be screened for by spreading bacteria on plates to form colonies in the presence of a limited number of *r*II phage particles (about 10^5 per plate). Under these conditions, most bacteria divide before encountering a phage particle and proceed to produce colonies. If the phage is inactive on a particular strain, the killing effect of the phage particles is negligible and most colonies develop smoothly. Mutant clones which permit the phage to multiply appear as nibbled colonies and are chosen for further testing. In looking for changes of type 2, the situation is reversed and the occasional solid colonies are chosen as suspects. Some of the variants isolated in this way are simply ones which have lost the block against all *r*II mutants (which can occur due to the loss of prophage λ) or ones which adsorb the phage poorly, but others show altered patterns of response specific for certain *r*II mutants.

In Table 4, a comparison is given of KT with a strain derived from it by treating with ethyl methane sulfonate and screening for resistance to *r*NT332, an ambivalent mutant of subset 1. KT-5 differs from KT in having lost the ability to support the multiplication of all ambivalent mutants of subset 1 but the remains similar to KT with respect to subset 2.

The behavior of a bacterial variant obtained by the converse procedure is illustrated in Figure 1 and in Table 5. KB-1 was obtained by treating KB with ethyl methane sulfonate and screening for sensitivity to *r*AP129, a mutant which is not active on either KB or KT. The result is a variant bacterium on which an entire set of *r*II mutants is active, thus revealing a third ambivalent subset. The mutants of this subset are identified in Figure 2 by stars. This subset numbers

TABLE 3

SPECIFICITY OF THE 5-FLUOROURACIL EFFECT ON THE ACTIVITY OF *r*II MUTANTS ON *E. coli* KB

	Transmission Coefficient on *E. coli* KB		
	Control	5FU	Response
Standard type			
r^+	0.95	0.88	−
Non-ambivalent *r*II mutants			
*r*131	0.000	0.000	0
*r*1272	0.000	0.000	0
54 mutants at different genetic sites			0
*r*N24	0.000	0.43	+
Ambivalent *r*II mutants of subset 1			
*r*HB118	0.002	0.65	+
*r*HB122	0.002	0.005	0
*r*C204	0.000	0.09	+
*r*N11	0.000	0.10	+
*r*EM64	0.000	0.78	+
*r*HB32	0.000	0.006	0
*r*HB35	0.000	0.37	+
*r*HB80	0.000	0.002	0
*r*HB129	0.000	0.31	+
*r*HB84	0.000	0.41	+
*r*2074	0.001	0.000	0
*r*EM84	0.000	0.47	+
*r*HB74	0.000	0.16	+
*r*NT332	0.000	0.63	+
*r*B94	0.000	0.27	+
*r*HB232	0.000	0.59	+

Measurements of transmission coefficient were made as described in the legend for Figure 3. The assays were made at t = 25 min at which time the maximum effect of 5-FU is reached.

TABLE 4

ALTERATION OF THE RESPONSE PATTERN OF STRAIN KT BY MUTATION

	Bacterial Host Strain	
	E. coli KT	*E. coli* KT-5
Standard type phage		
r^+	100.	100.
Non-ambivalent mutants		
*r*131	0.00	0.00
*r*1272	0.03	0.00
Ambivalent *r*II mutants of subset 1		
*r*HB118	98.	0.00
*r*HB122	42.	0.00
*r*C204	104.	0.00
*r*N11	127.	0.00
*r*EM64	99.	0.00
*r*HB32	19.	0.00
*r*HB35	100.	0.00
*r*HB80	6.3	0.00
*r*HB129	102.	0.00
*r*HB84	106.	0.00
*r*2074	20.	0.00
*r*EM84	79.	0.00
*r*HB74	72.	0.00
*r*NT332	72.	0.00
*r*B94	81.	0.00
*r*HB232	108.	0.00
Ambivalent *r*II mutants of subset 2		
*r*SN86	2.8	1.5
*r*HB309	3.0	1.5
*r*154	3.8	3.2
*r*NB3830	0.3	0.00
*r*N40	0.003	0.00
*r*HB33	1.1	0.4
*r*N89	0.6	0.4
*r*HB8	4.0	1.6
*r*DAP35	1.1	0.2

The table gives the burst size, relative to standard type phage, for various phage mutants on the two hosts. Bacterial strain KT-5 was isolated from KT by treating with ethyl methane sulfonate and screening for resistance to *r*NT332.

14 mutants, or approximately one per 25 sites. It remains to be seen how many subsets can be identified by an exhaustive survey.

Discussion.—The most striking aspect of the effects described is their extreme specificity. Since all *r*II mutants within a given cistron are defective in a unitary physiological function, the change in the host bacterium cannot concern its response to the function *per se*, or it would effect all the *r*II mutants of a cistron similarly. This does happen, for instance, if the bacterium loses its prophage λ, in which case all *r*II mutants become active. Bacterial variants are also known[17] in which the block against *r*II is partially removed, but again this applies to all *r*II mutants.

A review of the literature on suppression and a discussion of various proposed mechanisms has recently been given by Yanofsky and St. Lawrence.[1] One of their suggestions is that specific suppression might, in some cases, be explained by minor changes in the specificities of the system[11, 12] for activating amino acids and attaching them to acceptor RNA. Suppose, for example, that one of the activating enzymes were altered, so that it had some tendency to couple the wrong amino acid to a given acceptor RNA. The result could be the occasional insertion of an amino acid *a* in positions in a protein where amino acid *b* belongs. While such a fault in the translation of the genetic information would tend to reduce to some degree the activity of all enzymes, it could greatly increase the activity of an in-

TABLE 5

ALTERATION OF THE RESPONSE PATTERN OF STRAIN KB BY MUTATION

	Bacterial Host Strain	
	E. coli KB	*E. coli* KB-1
Standard type phage		
r^+	100.	100.
Non-ambivalent mutants		
*r*131	0.00	0.00
*r*1272	0.00	0.00
Ambivalent *r*II mutants of subset 3		
*r*AP129	0.2	45.
*r*AP80	0.2	6.9
*r*HB6	0.03	5.0
*r*106	0.1	2.2
*r*556	0.00	1.0
*r*263	0.02	7.6
*r*1948	0.5	108.
*r*N38	0.2	53.
*r*1221	0.05	5.8
*r*EM16	0.3	32.
*r*A84	1.3	97.
*r*SD80	0.00	7.9
*r*NT284	0.08	14.
*r*EM34	0.00	11.

Strain KB-1 was isolated from KB by treating with ethyl methane sulfonate and screening for sensitivity to *r*AP129. The table gives the burst size, relative to standard type phage, for various phage mutants on two hosts. The behavior of ambivalent mutants of subsets 1 and 2 is unaffected by the mutation from KB to KB-1.

active one in which the defect were due to a mutation of just the right kind, namely, one which has caused amino acid *b* to replace the normal *a*. A similar result might also be effected by modification of one of the acceptor RNA's. This theory would readily explain the specificity of suppression, as well as the case studied by Crawford and Yanofsky[13] in which a mutant synthesized an inactive protein, while the suppressed strain was shown to synthesize both the inactive variety and some of the active form.

As shown here, modification of the bacterium by mutation can lead to an altered response with respect to an entire subset of phage mutations, and the presence of 5FU leads to a similar effect. The high degree of coincidence between the specificity of action of 5FU and a bacterial mutation implies that closely related mechanisms may be involved in both cases. In the light of the observations of Naono and Gros on the alteration of proteins found in the presence of 5FU, it would seem not unlikely that the differences between strains KB and KT and the other bacterial mutants described may reside in slight differences in their mechanisms for translating genetic information. A prediction of the model would be that suppressor mutations, while specific in action on only certain mutations within a cistron, might nevertheless be capable of affecting some mutations in various other unrelated cistrons. This can be easily tested with *r*II mutants on strains of *E. coli* known to contain specific suppressors for one or another bacterial mutation.

In an attempt to obtain definitive evidence for this mechanism, the bacterial strains are being examined for changes in their activating enzymes and acceptor RNA. If changes in the specificity of activating enzymes or acceptor RNA can indeed be identified between bacteria differing by single mutations, experiments of the kind described in this paper may offer a promising approach to a study of the genetic control of the components of the cellular translation mechanism.

Differences in specificity for the acceptor RNA's and activating enzymes of different organisms are known to exist. Berg *et al.*[14] showed that if the acceptor RNA of *E. coli* is first saturated with methionine, using an activating enzyme from yeast, subsequent addition of activating enzyme from *E. coli* leads to attachment of still more amino acid. This experiment shows not only that the *E. coli* and yeast enzymes are different, but also that *E. coli* contains at least two types of RNA molecules capable of accepting methionine. For some of the other amino acids, the differences between *E. coli* and yeast are even more striking: the activating enzyme from either organism will attach amino acid only to its homologous acceptor RNA. This is known to be the case for at least leucine,[15] and for arginine and tyrosine.[16] It does not follow that the different acceptors for a given amino acid also behave differently in pairing with a given template. Nevertheless, in view of these facts, the possibility must be seriously considered that the rules for translation of a specific base sequence in hereditary nucleic acid into a corresponding sequence of amino acids in protein , i.e. the genetic code, may be different in different organisms.

Summary.—The set of *r*II mutants defective on one strain of *Escherichia coli* contains subsets having activity on other strains. The ability to grow, of the various subsets of the *r*II phage mutants, may be altered by mutation of the bacterium. Three distinct subsets have been identified so far, containing, respectively, one per 23, one per 39, and one per 25 *r*II sites. In addition, the phenotypes of certain *r*II mutants can be reversed by the action of 5-fluorouracil, an analog known to be incorporated into RNA. These phenomena are discussed in terms of possible modifications in the cellular mechanism for translating genetic information.

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TRANSPLANTATION IMMUNITY, IMMUNOLOGICAL TOLERANCE, AND CHICKEN × TURKEY INTERSPECIFIC HYBRIDS

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Although a high degree of tolerance of homografts of skin and other tissues can consistently be induced in a variety of avian and mammalian species by exposure of embryonic or neonatal hosts to homologous cells,[1, 2] nearly all attempts to confer tolerance in respect of grafts of *heterologous* origin have given very disappointing results in terms of the degrees of unresponsiveness obtained. However, the turkey-chicken species combination, studied in detail by Hašek and his associates using their elegant method of experimental embryonic parabiosis,[6] has proved to be exceptionally favorable for studies on tolerance of heterografts.[7–9]

The essential findings that have emerged from grafting and other tests conducted upon a restricted number of chickens and turkeys which had been parabiotically united in embryonic life,[7–9] or injected intravenously with heterologous blood at hatching,[1] are as follows: (1) Higher degrees of tolerance of skin heterografts—judged in terms of their survival times—are induced in turkeys than in their chicken parabiont partners. (2) In both species embryonic parabiosis is followed, at least initially, by red-cell chimerism, apparently as a consequence of a transient partial functional colonization of the host's marrow by hematopoietic cells of heterologous origin. (3) In turkeys a high degree of tolerance of skin heterografts is not necessarily accompanied by persistence of the state of red cell chimerism; tolerance of a skin heterograft may persist after the host has produced agglutinins in response to active immunization against the red cells of its former chicken parabiont partner; the tolerance induced may extend to skin heterografts from randomly selected chicken donors *other than* the original parabiotic partner and to *homografts* of turkey skin. (4) Chickens found to be only feebly tolerant of their turkey parabiont partners'[1] skin heterografts may nevertheless display high degrees of tolerance when subsequently challenged with *homografts* from randomly selected donors. The most interesting and provocative of these findings is, of course, that exposure of embryos to heterologous cells may render the hosts incapable of rejecting subsequent skin homografts from unrelated members of their *own* species.

In an attempt to account for the findings summarized above, Hašek and Hort[8] have dismissed as improbable the argument that there may be considerable overlap between the spectra of transplantation isoantigens that characterize the two species concerned. They postulated that, following prolonged exposure of turkey and chicken embryos to each others' cells, not only is the capacity of each to discriminate between individual antigens of the heterologous donor species impaired, but each also loses its ability to discriminate between transplantation isoantigens of its *own* species. The purpose of the grafting and other tests carried out upon two chicken × turkey interspecific hybrids (an interfamilial cross, taxonomically) to be described in this paper was to seek further evidence bearing upon Hašek and Hort's[8] interesting hypothesis.

Principle of the Experiments.—If, as these workers believe, exposure of the avian

embryos to the heterologous cellular antigens does deprive them of their normal ability to react against homografts, and to discriminate between grafts from different members of the heterologous donor species, then it might be argued that the incorporation of the genetic determinants of both turkey and chicken isoantigens (and probably of species-specific antigens as well) into the *same* genome, as must occur in interspecific hybrids, should also result in demonstrable failures to reject grafts from donors of either parental species. One must surely concede that each hybrid, like Hašek and Hort's parabionts, is thoroughly exposed from early life to a variety of what are normally recognized as isoantigens of each of its parental strains, in addition to species-specific factors.

Methods and Results.—The two adult hybrid birds available to us were male progeny of Beltsville Small White (BSW) turkey hens artificially inseminated with semen from male chickens of the Dark Cornish (DC) breed. Morphological evidence of the hybrid status of the birds, already published in detail,[14] includes the fact that their dark plumage resembles that of DC chickens, whereas BSW turkeys are recessive white in color. Furthermore, the results of serological studies on their red cells are in accordance with their being interspecific hybrids.[12]

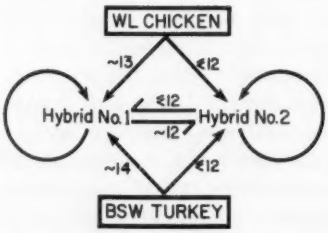

FIG. 1.—The pattern of skin grafting operations carried out on the two hybrids. The figures indicate the survival times of the various grafts in days. The closed circles indicate autografting operations.

(a) *Skin grafting tests:* Each hybrid was challenged with a well-trimmed (i.e., thin) graft of adult skin, about 1.5–2.0 cm in diameter, from each of the following: (i) a White Leghorn chicken, (ii) a BSW turkey, (iii) the other hybrid, and (iv) itself—as a control on the adequacy of the grafting technique employed. Recipient areas for their quartets of grafts were prepared on the saddle region and on the outer aspect of the hybrids' thighs.

The results of these grafting tests (see Fig. 1) were very clear-cut: With the exception of the control autografts, which healed in and survived in an exemplary manner, all other grafts soon became swollen and indurated and were destroyed within two weeks. Indeed, the responses of the hybrids to the three types of alien skin grafts were similar to those of the BSW turkeys to chicken skin and of WL chickens to BSW skin.

(b) *"Grafting" tests with hybrid blood:* To obtain the maximum possible information concerning the ability of a hybrid to discriminate between the antigens of each of its parent species, either: (i) many hybrids must receive grafts from one or other of a whole battery of donors, or (ii) as in the present situation where only two of these birds were available, some other means must be sought to test their capacity to react immunologically against the various tissue antigen combinations from a wide range of donors. The placing of multiple grafts upon each host was impracticable because of their delicate constitution.

The test system employed required only a few ml of freshly drawn and heparinized blood from each hybrid. Its principle is simple and depends upon the elegant analytical studies of Simonsen,[15] and Cock and Simonsen.[5] When adult homologous immunologically competent cells, e.g., spleen cells or simply lymphoid cells present in whole blood, are inoculated onto the chorioallantoic membrane of chick embryos, or are injected directly into the blood stream, these cells proliferate and react against the foreign isoantigens of their hosts. Among the diagnostic features of this graft-versus host-reaction is the appearance of small whitish nodules in the substance of the chorioallantoic membrane[3] and profound enlargement of the embryo's spleen[15]—the latter lending itself to quantitative appraisal. Mitchison[13] has shown that a similar reaction occurs when immunologically competent cells of turkey origin are inoculated into chicken embryos.

Aliquots of 0.2 ml of this blood from each hybrid were placed upon the "dropped" chorioallantoic membranes of a series of 10-day WL chicken embryos. The eggs were then incubated for an additional 9 days after which their shells were removed, the membranes carefully inspected, and

the embryos' spleens excised and weighed. Controls were provided by weighing the spleens of uninjected 19-day-old WL embryos and those of WL embryos of similar age whose membranes had been dropped on the tenth day of incubation and inoculated with 0.2 ml of physiological saline. For comparative reasons one series of 10-day WL embryos was inoculated with 0.2 ml of heparinized blood from an adult BSW turkey donor.

The various tests conducted, together with their results, are summarized in Table 1. Clearly

TABLE 1

SPLEEN WEIGHTS OF 19-DAY-OLD WHITE LEGHORN EMBRYOS INOCULATED WITH 0.2 ML ADULT TURKEY OR TURKEY × CHICKEN INTERSPECIFIC HYBRID BLOOD NINE DAYS PREVIOUSLY

No. of Inoculum	No. of embryos	Wts. of spleens removed from embryos (mg) <25	25–40	40–55	>55	Mean spleen wt. and range (mg)
None	9	9	...	...	...	14.2 (10.2–17.6)
0.2 ml saline	8	8	...	..	...	15.3 (11.3–23.8)
0.2 ml BSW turkey blood	19	4	4	2	9	55.7 (18.7–127.9)
0.2 ml blood from hybrid #1	19	...	3	14	2	38.8 (25.4–57.6)
0.2 ml blood from hybrid #2	11	1	6	4	0	35.8 (23.6–46.8)

hybrid blood is not inferior to turkey blood in its ability to react against and produce splenomegaly in nearly every WL embryo into which it is inoculated. Further, with both types of blood, whitish nodules of variable diameters were almost invariably observed on the chorioallantoic membranes of the hosts at autopsy. In light of Simonsen and Jensen's[16] investigation of the splenomegaly produced by inoculating homologous lymphoid cells into infant mice, it seems reasonable to suppose that the considerable variability in the weights of the spleens of the WL embryos reflects, at least in part, different degrees of immunogenetic disparity between the "target" WL embryos and the hybrid's lymphoid cells.

Discussion and Conclusions.—From these results it seems reasonable to conclude that hybridization of turkeys with chickens does not result in any demonstrable loss of ability on the part of the hybrids to discriminate between and react against skin grafts of various genetic constitutions from *either* parental species, or from other hybrids of similar origin.

Unless the argument that the immunological status of our hybrid birds is comparable to that of Hašek and Hort's tolerant birds is based upon false premises, the present findings are inconsistent with their interpretation of the apparent lack of specificity in graft compatibility, following interspecific parabiosis.

An alternative hypothesis, which accounts for all the results and which does not necessitate discarding the concept of the strict specificity of tolerance is as follows: Chickens and turkeys are fairly closely related taxonomically, belonging to the Order Galliformes—the fact that interspecific hybrids can be produced *strongly* substantiates this—and, although each probably possesses some distinctive species-specific antigens, some of which are present on their erythrocytes, it is not unlikely that each possesses a number of antigens, including transplantation isoantigens, which may be related both in immunological specificity and by genetic derivation.[10,11,17] Some of the points made by Cinader[4] in a recent consideration of specificity and inheritance of antibody response are particularly pertinent here. In an immunological sense, therefore, turkeys and chickens behave like two widely diverse, long isolated populations of a single species. The fact that only a small number of the subjects manifest high degrees of tolerance of skin heterografts, following interspecific embryonic parabiosis, may indicate that such tolerant birds constitute a highly selected group. Their susceptibility to being made tolerant of their partners' tissues may have originated from the possession of a particular

genotype—probably a highly heterozygous one—so that they possess more of the important transplantation antigens segregating in their own species than birds that failed to become tolerant of heterografts.

In addition to the broad spectrum of isoantigens to which these birds may be genetically unresponsive, must be superimposed the spectrum of antigens (including isoantigens common to both turkeys and chickens) of the individual of the *other* species to which they were rendered immunologically tolerant as a consequence of parabiosis. It is by no means inconceivable, therefore, that at the time of hatching, these birds were incapable of reacting against all, or at least a high proportion, of the important transplantation antigens segregating in their *own* stock of origin, especially if this comprised a relatively small, closed population.

One would, of course, expect the spectrum of parental species antigens, to which interspecific hybrids are genetically unresponsive, to be much narrower than in members of either species made immunologically tolerant of the other. A hybrid can only be genetically unresponsive to the antigens determined by its own two sets of chromosomes (including "hybrid" antigens)—one from each parent; whereas a tolerant bird can, in theory, be unresponsive to all the antigens determined by 4 sets of chromosomes—its own 2 plus those of its parabiont partner.

It seems appropriate to utter a caution against the possible dangers of assuming *a priori* the existence of a sharp distinction between antigens responsible for isoimmune reactions, including the homograft reaction, on the one hand and those responsible for heteroimmune reactions, including the rejection of heterografts, on the other. With combinations of widely unrelated species the distinction is probably valid, but at least with some closely related species, such as turkeys and chickens (and probably horses and donkeys and other species combinations where F_1 hybrids can be produced), the forces responsible for their evolutionary dichotomy may not have exerted much influence on the nature or properties of segregating genetic determinants of isoantigens that each has inherited from some common ancestral stock. The results of Irwin and Miller's[11] recent studies on the red-cell antigens of different species of Columbidae are particularly pertinent here.

Summary.—Evidence is presented that adult turkey × chicken interspecific hybrids promptly reject tissue grafts from donors of both parental species and from other turkey-chicken hybrids. In addition, whole blood from these animals inoculated onto the chorioallantoic membranes of chick embryos consistently produces a high degree of splenomegaly. These findings indicate that the capacity of hybrids to react against the normal transplantation isoantigens of the parental species is fully developed. The results are discussed in relation to the apparent lack of specificity of immunological tolerance of tissue grafts alleged to occur following interspecific embryonic parabiosis.

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*AN ALGEBRA OF ADDITIVE RELATIONS**

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1. *Introduction.*—If A and B are modules over a fixed ring, a homomorphism $\alpha: S \to B/L$ of a submodule S of A into a quotient module of B may be regarded as a "many-valued" homomorphism f of A onto B; it is defined only on elements of S, and assigns to each s all the elements $f(s)$ in the coset $\alpha(s)$ of B. It may also be construed as the binary relation consisting of all the pairs $(s, f(s))$ for s in S; this relation turns out to be additive, and any additive binary relation on A and B is such a homomorphism.

Such additive relations occur more frequently than is usually realized. In algebraic topology, a (primary) cohomology operation such as the Steenrod square is a natural homomorphism $Sq^i: H^n(X) \to H^{n+i}(X)$ defined for the mod 2 cohomology of all topological spaces X. Recent studies[1] indicate the importance of certain secondary cohomology operations, which are homomorphisms defined on suitable submodules of $H^n(X)$ with values in appropriate quotient modules of $H^{n+i}(X)$—the submodules in question being normally the kernels of some primary cohomology operations. Hence, the secondary cohomology operations are additive relations.

The composite of two additive relations is another such. The inverse of a homomorphism $\beta: B \to A$ is an additive relation β^{-1} which assigns to each element a in the image of β all the elements in the inverse image of a. A composite of homomorphisms and inverses of homomorphisms is thus an additive relation which often turns out to be an honest single-valued homomorphism. This type of "inverse composite" (terminology of S. Eilenberg) occurs in many of the basic constructions

of homological algebra. The "connecting homomorphism" is typical. Given a map (α, β, γ) of one short exact sequence into another

$$\begin{array}{ccccccccc} 0 & \longrightarrow & A & \xrightarrow{\kappa} & B & \xrightarrow{\sigma} & C & \longrightarrow & 0 \\ & & \downarrow{\scriptstyle\alpha} & & \downarrow{\scriptstyle\beta} & & \downarrow{\scriptstyle\gamma} & & \\ 0 & \longrightarrow & A' & \xrightarrow{\kappa'} & B' & \xrightarrow{\sigma'} & C' & \longrightarrow & 0 \end{array}$$

the inverse composite $(\kappa')^{-1}\beta\sigma^{-1}$ is an additive relation on C to A' which induces the connecting homomorphism Δ: (Ker γ) $\rightarrow$ (Coker α) which occurs in the standard exact sequence

$$\text{Ker } \alpha \rightarrow \text{Ker } \beta \rightarrow \text{Ker } \gamma \xrightarrow{\Delta} \text{Coker } \alpha \rightarrow \text{Coker } \beta \rightarrow \text{Coker } \gamma.$$

This Δ is the model for the usual connecting homomorphisms (e.g., for a short exact sequence of complexes).

D. A. Puppe (unpublished lectures) has observed that spectral sequences can be treated efficiently using additive relations. Usually a spectral sequence is a sequence (E_r, d_r) of modules E_r with differential $d_r (r = 2, 3, \ldots)$ such that $H(E_r, d_r) = E_{r+1}$. Thus each E_r is a subquotient of E_2 (a submodule of a quotient module of E_2), so each d_r may be regarded as an additive relation on E_2. Call an additive relation d: $E \rightarrow E$ a *differential relation* if, in the obvious sense, Image $d \subset$ Kernel d. Then a (cohomology) spectral sequence may be described as a bigraded module $E = E_2$ equipped with a family d_r of differential relations d_r: $E \rightarrow E$ of bidegree $(r, 1-r)$ such that $d_{r+1}0 = d_r E$ and $d_{r+1}^{-1}E = d_r^{-1}0$; these latter conditions insure that $H(E_r) = E_{r+1}$. The description of the spectral sequence of an exact couple or of a filtered differential module may also be conveniently formulated via composites of additive relations.

Starting with any abelian category (e.g., the category of all R-modules), one may define the additive relations $f: A \rightarrow B$ to be the submodules of the direct sum $A \oplus B$ and establish formal properties of composites, inverses, etc. sufficient to carry out the necessary proofs, such as the proof of the exactness of the connecting homomorphism sequence quoted above. This amounts to a derivation of the properties of many-valued homomorphisms from those of single-valued homomorphisms, as codified in the notion of an abelian category.[2] Here, we take the opposite point of view. We regard the additive relations as basic and set up suitable formal axioms valid in the category of all additive relations. This has several advantages. First, it has long been known that the theory of abelian categories formally subsumes both the composition properties of homomorphisms and the lattice properties of submodules. However, the construction of submodules in an abelian category is quite indirect, in that the submodules must be described as equivalence classes of monomorphisms. In the category of additive relations, the lattice order properties are present from the start, along with the composition properties. Secondly, this consideration suggests other algebraic studies; for example, the set of additive relations on A, A is a lattice ordered semi-group with an involution in which every element is regular in the sense of von Neumann. It is to be expected that semigroups with such structures will repay further study.

Recall that a *category* is a class of "objects" with a function assigning to each pair of objects A, B a set hom (A, B) of "morphisms" such that to $f \in \text{hom}\ (A, B)$ and $g \in \text{hom}\ (B, C)$ there is defined a composite $gf \in \text{hom}\ (A, C)$. This composition is associative (when defined), and to each A there is $1_A \in \text{hom}\ (A, A)$ with $f1_A = f$, $1_B f = f$.

2. *Additive Relations.*—Our standard model $\mathfrak{M}$ is the category with objects all (left) modules A, B, ... over a fixed ring R, and with morphisms $f: A \to B$ all additive relations, that is, all submodules of the direct sum $A \oplus B$. Each f is thus a set of ordered pairs (a, b) closed under addition and under multiplication by elements of R. The *converse* of f, written $f^\sharp$, is the additive relation $f^\sharp: B \to A$ consisting of all pairs (b, a) such that $(a, b) \in f$. If $g: B \to C$, the composite (the relative product) $gf: A \to C$ is the set of all those pairs (a, c) such that there is a $b \in R$ with $(a, b) \in f$ and $(b, c) \in g$. By $\text{Rel}(A, B)$ we denote the set of all additive relations $f: A \to B$; with the usual inclusion relation for submodules, $\text{Rel}(A, B)$ is a modular lattice.

THEOREM 2. *For each additive relation f, $ff^\sharp f = f$.*

Proof: Trivially $f \subset ff^\sharp f$. Conversely, $(a, b) \in ff^\sharp f$ implies that there are $b' \in B$ and $a' \in A$ with $(a, b') \in f$, $(b', a') \in f^\sharp$, and $(a', b) \in f$, hence $(a, b') - (a', b') = (a - a', 0) \in f$, hence $(a, b) = (a', b) + (a - a', 0) \in f$, so $ff^\sharp f \subset f$.

A *subquotient* of a module A is a module of the form S/K, where S and K are submodules with $K \subset S \subset A$. A subquotient may be described as a submodule of the quotient module A/K, or as a quotient module of the submodule $S \subset A$.

For each $f: A \to B$ introduce the usual submodules

$$\text{Def}\, f = [a \mid (\exists b),\ (a, b) \in f] \subset A, \qquad \text{Im}\, f = \text{Def}\, f^\sharp \subset B, \tag{2.1}$$

$$\text{Ker}\, f = [a \mid (a, 0) \in f] \subset A, \qquad \text{Ind}\, f = \text{Ker}\, f^\sharp \subset B. \tag{2.2}$$

Here Ind f means "the indeterminacy of f" and $\text{Ker}\, f \subset \text{Def}\, f$. Moreover, f induces an isomorphism θ: $\text{Def}\, f/\text{Ker}\, f \cong \text{Im}\, f/\text{Ind}\, f$, given by $\theta(a + \text{Ker}\, f) = b + \text{Ind}\, f$ for each $(a, b) \in f$. Conversely, a subquotient S/K of A, a subquotient T/L of B and an isomorphism θ: $S/K \cong T/L$ determines a unique additive relation $f: A \to B$ which has $S = \text{Def}\, f \supset K = \text{Ker}\, f$, $T = \text{Im}\, f \supset L = \text{Ind}\, f$ and which induces θ as above. Likewise, f induces an ordinary homomorphism $\text{Def}\, f \to B/\text{Ind}\, f$ with kernel Ker f and image Im f/Ind f. Conversely, each homomorphism of a submodule of A to a quotient module of B is so induced by a unique $f \in \text{Rel}\ (A, B)$.

THEOREM 2.2 *An additive relation $u: A \to A$ with $u^\sharp = u = u^2$ has the form*

$$u = u_{S,\,K} = [(s, s + k) \mid s \in S, k \in K] \tag{2.3}$$

for submodules $K \subset S \subset A$. Conversely, submodules $K \subset S \subset A$ determine an additive relation (2.3) with $u^\sharp = u = u^2$.

Proof: If $u^\sharp = u$, set $S = \text{Def}\, u = \text{Im}\, u$ and $K = \text{Ker}\, u = \text{Ind}\, u$. If $(a, c) \in u$, then $(c, a) \in u^\sharp = u$, hence $(a, a) \in u^2 = u$. Thus $s \in \text{Def}\, u$, $k \in \text{Ker}\, u$ implies (s, s) and $(s, s + k) \in u$. Moreover, $(a, c) \in u$ implies $(a, c) - (a, a) = (0, c - a) \in u$, so $a \in S$, $c - a \in K$. Thus $u = u_{S,\,K}$ as given in (2.3). Conversely, $u_{S,\,K}$ is a symmetric idempotent.

By this result, the subquotients of A are in one-to-one correspondence with the symmetric idempotents in $\text{Rel}(A, A)$; we say that the subquotient S/K is "represented" by $u = u_{S,\,K}$. For example,

$$f^\sharp f = [(a, a') \,|\, a, a' \in \text{Def } f,\ a - a' \in \text{Ker } f] \tag{2.4}$$

represents Def f/Ker f. Moreover, if $1_A : A \to A$ is the identity relation (all pairs (a, a) for $a \in A$), then

$$f^\sharp f \cap 1_A = [(a, a) \,|\, a \in \text{Def } f] \tag{2.5}$$

represents the submodule Def f.

3. *Initial Axioms; Idempotents.*—A *partially ordered category* $\mathfrak{B}$ will be a category, with Rel (A, B) as the set of morphisms $f : A \to B$, satisfying three groups of axioms, all valid in the standard model $\mathfrak{M}$ of §2. The first group reads

(I-a) *To each $f : A \to B$ there is a unique $f^\sharp : B \to A$ with*

$$f^{\sharp\sharp} = f = ff^\sharp f, \qquad (fg)^\sharp = g^\sharp f^\sharp.$$

(I-b) *Each Rel(A, B) is a modular lattice under a partial order relation $\subset$ such that, for f, g: $A \to B$,*

$$g \subset f \quad \text{implies} \quad g^\sharp \subset f^\sharp,\ gh \subset fh.$$

Here we are to read "$gh \subset fh$ when the composites are defined"; that is, when $h : C \to A$ for some object C of the category. We write $\cap$ and $\cup$ for the intersection and union in this lattice. These axioms (like the later ones) are self-dual in the lattice sense: reversing inclusion and replacing $\cap$ by $\cup$ (and preserving the order of composition) leave valid theorems valid. Elementary lattice arguments now prove $1_A{}^\sharp = 1_A$ and

$$(f \cap g)h \subset fh \cap gh, \qquad k(f \cap g) \subset kf \cap kg, \tag{3.1}$$

$$(f \cup g)^\sharp = f^\sharp \cup g^\sharp, \qquad (f \cap g)^\sharp = f^\sharp \cap g^\sharp, \tag{3.2}$$

for $f \to f^\sharp$ is by axiom (I-b) a lattice isomorphism.

A *symmetric idempotent* u is an element of some Rel (A, A) such that $u^\sharp = u = u^2$; equivalently, by (*I-a*), a symmetric idempotent is a morphism u of the form $u = ff^\sharp$ for some f.

THEOREM 3.1. *The composite of two symmetric idempotents is an idempotent.* For

$$uv = uv(uv)^\sharp uv = uvv^\sharp u^\sharp uv = uvuv.$$

THEOREM 3.2. *The symmetric idempotents on A form a sublattice of Rel (A, A).*

Proof: Set $f = u \cup v$. Then $f \supset u$ implies $f^2 \supset u^2 = u$; hence $f^2 \supset u \cup v = f$, while $f^\sharp = f$ by (3.2). But $f^2 \supset f$ gives $f^3 \supset f^2 \supset f = ff^\sharp f = f^3$, whence $f^2 = f$ is idempotent. Dually, $u \cap v$ is a symmetric idempotent.

In the terminology (2.3) of the model, the union $u_{D,K} \cup u_{D',K'}$ is $u_{D \cup D',\, K \cup K'}$. Thus our axioms at once give the lattice of subquotients of each object A of the category. It contains the lattices of subobjects as follows:

THEOREM 3.3. If $s \subset 1_A : A \to A$, then s is a symmetric idempotent. *If also $t \subset 1_A$, then $s \cap t = st$; hence $st = ts$.*

By (I-a), $s = ss^\sharp s \subset ss^\sharp 1_A = ss^\sharp \subset s1_A = s$. Hence $s = ss^\sharp$. Therefore $s^2 = ss^\sharp ss^\sharp = ss^\sharp$ by (*I-a*), so $s = s^2$ and $s^\sharp = s^{\sharp\sharp} s^\sharp = s$. If also $t \subset 1$, then $st \subset s \subset 1$, and thus $st \subset s \cap t$. Conversely, $r = s \cap t$ is a symmetric idempotent by Theorem 3.2, and $r \subset s$, $r \subset t$ give $r = rr \subset rt \subset st$, so $s \cap t \subset st$, giving the desired equality.

The treatment of quotients of A (all $q \supset 1_A$) is dual. Note also

THEOREM 3.4. *If u and v are symmetric idempotents in $Rel(A, A)$,*

$$u \cap v \subset uv \subset u \cup v. \tag{3.3}$$

Proof: Since $w = u \cap v$ is idempotent, $w = w^2 \subset uv$, and dually.

Each $f: A \to B$ determines the idempotents $f^\sharp f$ and $ff^\sharp$, which in the model represent the subquotients Def f/Ker f and Im f/Ind f, respectively. Call f a *graph* if $f^\sharp f \supset 1$ and $ff^\sharp \subset 1$; in the model, a graph is exactly a relation which is a single-valued homomorphism, with Def $f = A$ and Ind $f = 0$. It follows from the axioms that the composite of two graphs is a graph, so the graphs form a subcategory (with the same objects as $\mathfrak{R}$). Similarly, the monomorphisms, characterized by $f^\sharp f = 1$, $ff^\sharp \subset 1$, constitute a subcategory. The formalism contains the maps induced by each f on subquotients.

THEOREM 3.5. *Each $f: A \to B$ induces an order-preserving map $u \to fuf^\sharp$ of the symmetric idempotents of A to those of B, with*

$$f(u \cup v)f^\sharp \supset (fuf^\sharp) \cup (fvf^\sharp) \tag{3.4}$$

and dually. If f is a graph, $u \subset 1_A$ implies $fuf^\sharp \subset 1_B$ (so the map carries subobjects to subobjects).

Proof: The symmetry of u implies that of $fuf^\sharp$ by (*I-a*). Since $f^\sharp fu$ is idempotent by Theorem 3.1,

$$fuf^\sharp\, fuf^\sharp = f(f^\sharp fu)(f^\sharp fu)f^\sharp = f(f^\sharp fu)f^\sharp = fuf^\sharp,$$

and $fuf^\sharp$ is idempotent. The map $u \to fuf^\sharp$ preserves order by (*I-b*), and (3.4) follows from the dual of (3.1).

For the sequel, it is convenient to set

$$Df = f^\sharp f \cap 1 \qquad K'f = f^\sharp f \cup 1. \tag{3.5}$$

In the model, Df represents Def f as a relation $Df \subset 1_A$ and $K'f$ represents A/Ker f as a relation $K'f \supset 1_A$. Also $K'f^\sharp$ represents the indeterminacy of f.

4. *Inclusion Axioms.*—The axioms of our first group are natural but do not adequately express the intimate relation between composition and the partial order: in the model (Theorem 4.5), the partial order is entirely determined by the composition and the sets $S(A) = [s \mid s \subset 1_A]$ and $Q(A) = [q \mid q \supset 1_A]$. We now add a second group of rather curious axioms, all valid in the standard model.

(II-*a*) $hh^\sharp \subset K'f$ *implies* $(f \cap g)h \supset fh \cap gh$,

(II-*b*) $hh^\sharp \supset Df$ *implies* $(f \cup g)h \subset fh \cup gh$,

(II-*c*) $f, g \in Rel\,(A, B)$ *implies* $f^\sharp g \cap 1_A \subset f^\sharp f \subset f^\sharp g \cup 1_A$,

(II-*d*) $g \subset f$, $Dg = Df$, *and* $K'(g^\sharp) = K'(f^\sharp)$ *imply* $g = f$.

Lattice duality still holds. The first axiom, with (3.1), gives a sufficient condition for the distributive law $(f \cap g)h = fh \cap gh$—the model indicates that a hypothesis such as $hh^\sharp \subset K'f$ is necessary for the law. Since $1 \subset K'f$, this law holds whenever h is a graph. The involution $^\sharp$ on (II-*a*) yields

$$h^\sharp h \subset K'(f^\sharp) \quad \text{implies} \quad h(f \cap g) = hf \cap hg, \tag{4.1}$$

and dually. Moreover, these axioms show that equality holds in (3.4) when f is a graph. Indeed f a graph gives $f^\sharp f \supset 1$ and hence, by distributivity (II-*b*), $(fu \cup v)f^\sharp = fuf^\sharp \cup fvf^\sharp$. These distributive laws also prove

$$f(f^\sharp f \cap 1) = f = f(f^\sharp f \cup 1),\ (gg^\sharp \cap 1)g = g = (gg^\sharp \cup 1)g. \tag{4.2}$$

The first identity gives a characteristic property of $Df = f^\sharp f \cap 1$; indeed we can describe Df as the l.u.b. of those $s \subset 1$ for which $fs = f$:

THEOREM 4.1. *For a symmetric idempotent u with fu defined, $fu = f$ if and only if $f^\sharp f \cap 1 \subset u \subset f^\sharp f \cup 1$.*

The proof makes essential use of the innocent but powerful axiom (II-c). First suppose $fu = f$ for some $f \in \mathrm{Rel}(A, B)$. Then $u, f^\sharp f \in \mathrm{Rel}(A, A)$, so by (II-$c$) $u^\sharp f^\sharp f \cap 1 \subset u^\sharp u \subset u^\sharp f^\sharp f \cup 1$. With $u^\sharp f^\sharp = f^\sharp$ and $u^\sharp u = u$, this gives the asserted inclusion. The converse implication follows by (4.2). The same argument proves

$$f \supset fu \quad \text{if and only if} \quad u \subset ff^\sharp \cup 1, \tag{4.3}$$

and dually. Applying $^\sharp$ to (II-c) gives

$$f, g \in \mathrm{Rel}(A, B) \quad \text{implies} \quad g^\sharp f \cap 1_A \subset f^\sharp f \subset g^\sharp f \cup 1_A. \tag{4.4}$$

THEOREM 4.2. *If u is a symmetric idempotent,*

$$u = u(u \cap 1) = (u \cap 1)u = u(u \cup 1) = (u \cup 1)u = (u \cup 1)(u \cap 1) = (u \cap 1)(u \cup 1). \tag{4.5}$$

Conversely, if $u = sq$ with $s \subset 1 \subset q$, then $sq = qs$, $s = u \cap 1$, and $q = u \cup 1$.

Proof: The first four equalities follow by (4.2). Then $(u \cup 1) \supset u \supset (u \cap 1)$ implies $(u \cup 1)(u \cap 1) \supset u(u \cap 1) = u = (u \cup 1)u \supset (u \cup 1)(u \cap 1)$, which gives the rest. Conversely, $u = sq$ with $q \supset 1$ implies $s \subset u \cap 1$, while by (II-c), $s^\sharp q \cap 1 \subset s^\sharp s$, so $u \cap 1 \subset s$ and $u \cap 1 = s$. Dually $u \cup 1 = q$, while $sq = qs$ by (4.5).

The domain of definition of a relation has the following properties, all clearly valid in the standard model:

THEOREM 4.3. *For gf defined, $D(gf) \subset Df$, while for $s \subset 1$, $q \supset 1$,*

$$D(qf) = D(f), \qquad D(fs) = (Df) \cap s. \tag{4.6}$$

Proof: By definition, $Df = f^\sharp f \cap 1$. For $h = gf$, $h(Df) = h$ by (4.2), and Df is a symmetric idempotent, so by Theorem 4.1, $Dh \subset Df$. $D(qf) = f^\sharp q^\sharp qf \cap 1 = f^\sharp qf \cap 1 \supset f^\sharp f \cap 1 = Df$, with the reverse inclusion given by the previous result, so $D(qf) = Df$. As for the last equality, $D(fs) = s^\sharp f^\sharp fs \cap 1 = s(f^\sharp f)s \cap 1 = (f^\sharp f \cap 1) \cap s$, where the last step follows by

LEMMA 4.4. *If $s \subset 1_A$ and $u \in Rel(A, A)$ is a symmetric idempotent,*

$$(sus) \cap 1 = (u \cap 1) \cap s = u \cap s. \tag{4.7}$$

Proof: The second equality is immediate. By Theorem 3.3,

$$(u \cap 1) \cap s = (u \cap 1)s = s(u \cap 1)s \subset sus,$$

so $u \cap s \subset (sus) \cap 1$. Now, $s, us \in \mathrm{Rel}(A, A)$, so (II-$c$) gives $sus \cap 1 = s^\sharp us \cap 1 \subset s^\sharp s = s$, which with $sus \subset u$ gives the converse inclusion.

Note that the dual of Theorem 4.3 reads, for $K'f = f^\sharp f \cup 1$,

$$K'(gf) \supset K'f, \qquad K'(sf) = K'(f), \qquad K'(fq) = (K'f) \cup q. \tag{4.8}$$

In the model $g \subset f$ means that the domain of g is included in that of f, and that on the domain, the indeterminacy of g is included in that of f. In words, f cut

down to the domain of g equals g with indeterminacy "blown up" to that of f; in symbols $(K'f^\sharp)g = f(Dg)$. This property follows from the special case contained in Axiom (II-d).

THEOREM 4.5. *For $g, f \in Rel(A, B)$ the following properties are all equivalent:*

(*i*) $g \subset f$,
(*ii*) $(K'f^\sharp)g = f(Dg)$,
(*iii*) *There exist* $q \supset 1_B$, $s \subset 1_A$ *with* $qg = fs$,
(*iv*) *There exist* q, s, *with* $qg = fs$, $qf = f$, $gs = g$,
(*v*) $f^\sharp g \supset g^\sharp g \cap 1$ *and* $gf^\sharp \subset ff^\sharp \cup 1$.

Proof: Given (*i*), $Dg \subset Df$ and $K'g^\sharp \subset K'f^\sharp$. Set $s = Dg$ and $q = K'f^\sharp$. Then $qg = qgs \subset qfs = fs$, while by (4.6) and (4.8), $D(fs) = (Df) \cap s = Df \cap Dg = Dg = D(qg)$ and similarly $K'[(fs)^\sharp] = K'[(qg)^\sharp]$. Hence, by axiom (II-$d$), $qg = fs$, as in (*ii*). Trivially, (*ii*) implies (*iii*). Given (*iii*), $Dg = D(qg) = D(fs) = Df \cap s$, so $Dg \subset s$, and by Theorem 4.1 $gs = g$. Similarly $qf = f$, which gives (*iv*). Given (*iv*),

$$g = gs \subset qgs = fss = fs \subset f,$$

as in (*i*). Given (*i*), $f^\sharp g \supset g^\sharp g$, $gf^\sharp \subset ff^\sharp$ which is (*v*). Given (*v*),

$$g = g(g^\sharp g \cap 1) \subset g(f^\sharp g \cap 1) \subset gf^\sharp f \subset (ff^\sharp \cup 1)f = f,$$

where the middle inclusion follows by (II-c).

This theorem has some curious consequences, to wit:

COROLLARY 4.6. *For $g \subset f \in \mathrm{Rel}(A, B)$,*

$$fg^\sharp g = ff^\sharp g, \qquad gg^\sharp f = gf^\sharp f, \tag{4.9}$$

$$(g^\sharp f)(g^\sharp f) = g^\sharp f, \qquad (f^\sharp g)(f^\sharp g) = f^\sharp g. \tag{4.10}$$

Proof: By (*iv*) of the Theorem, $ff^\sharp g = f(qf)^\sharp g = ff^\sharp qg = ff^\sharp fs = fs = qg = qgg^\sharp g = fsg^\sharp g = f(gs)^\sharp g = fg^\sharp g$. This result for $g^\sharp \subset f^\sharp$ gives the second equality of (4.9). Finally, $g^\sharp f = g^\sharp qf = (qg)^\sharp f = (fs)^\sharp f = sf^\sharp f$ is the composite of two symmetric idempotents, hence is idempotent by Theorem 3.1. This, with converse, is (4.10).

The force of (II-c) is indicated by the following result, which is easily checked on the standard model and which will be used in the next section:

THEOREM 4.7. *For $f, g \in Rel(A, B)$, $1 \cap f^\sharp g = 1 \cap (f \cap g)^\sharp (f \cap g)$.*

Proof: By (3.2), $(f \cap \varsigma)^\sharp = f^\sharp \cap g^\sharp$. The hypotheses of the distributive laws (II-a) and (4.1) hold so as to give

$$\begin{aligned}(f^\sharp \cap g^\sharp)(f \cap g) &= [(f^\sharp \cap g^\sharp)f] \cap [(f^\sharp \cap g^\sharp)\varsigma] \\ &= f^\sharp f \cap g^\sharp f \cap f^\sharp g \cap g^\sharp g.\end{aligned}$$

By (II-c) and (4.4), $1 \cap f^\sharp g \subset f^\sharp f$, and $1 \cap f^\sharp g \subset g^\sharp g$. Since $1 \cap f^\sharp g$ is symmetric by Theorem 3.3, $1 \cap f^\sharp g = 1 \cap g^\sharp f \subset g^\sharp f$. Hence,

$$1 \cap f^\sharp g \subset 1 \cap f^\sharp f \cap g^\sharp f \cap f^\sharp g \cap \varsigma^\sharp g.$$

The converse inclusion is immediate, hence the desired result.

5. *Universal Bounds.*—Each submodule $S \subset A$ determines a corresponding quotient A/S, and conversely. The formulation of this correspondence depends

in the model on the null relation $\Omega \in \mathrm{Rel}(A, B)$, consisting only of the pair (0, 0), and the universal relation $\mho$, consisting of all pairs (a, b) for $a \in A$, $b \in B$. For these we assumed the following elementary axioms:

(III-1) *For each pair of objects A, B there exist $\Omega = \Omega_B{}^A$, $\mho = \mho_B{}^A$ in Rel(A, B) such that $f \in \mathrm{Rel}(A, B)$ implies $\Omega \subset f \subset \mho$,*

(III-2) $\Omega\mho = \Omega\mho, \qquad \Omega\Omega = \Omega, \qquad \mho\mho = \mho,$

(III-3) $\Omega\mho\Omega = \Omega, \qquad \mho\Omega\mho = \mho.$

Here the first equation of (III-2) reads in full: For four objects A, B, C, D, $\Omega_B{}^C\mho_C{}^A = \Omega_B{}^D\mho_D{}^A$. (It actually suffices to assume the axiom only in the case $B = A = D$, deducing the more general case.) The other axioms are to be read similarly.

Clearly $\Omega^\sharp = \Omega$, $\mho^\sharp = \mho$. We also define

$$0 = 0_B{}^A = \Omega_B{}^B\mho_B{}^A, \qquad 0^\sharp = \mho\Omega; \tag{5.1}$$

in the model 0 is the graph of the zero homomorphism. By (III-3), the various composites are given by

$$0\Omega = \Omega = 00^\sharp, \qquad 0\mho = 0 = 00 = \Omega 0, \qquad 0^\sharp 0 = \mho = 0^\sharp\mho = \mho 0. \tag{5.2}$$

For any f, (III-1) and the other axioms give

$$\Omega f\Omega = \Omega, \qquad \mho f\mho = \mho, \qquad 0f0 = 0, \qquad 0f0^\sharp = \Omega. \tag{5.3}$$

LEMMA 5.1. *For $g \in Rel(A, B)$, $\Omega_B{}^B g = g \cap 0_B{}^A$.*

Proof: Since $\Omega g \subset 1g = g$ and $\Omega g \subset \Omega\mho = 0$, we have $\Omega g \subset g \cap 0$. For the converse inclusion, set $h = g \cap 0$. Then $h \subset g$, $h \subset 0$ give $h = hh^\sharp h \subset hh^\sharp g \subset 00^\sharp g = \Omega\mho\Omega g = \Omega g$, q.e.d.

As a consequence of this lemma and its dual, note that

$$0 \cap 1 = \Omega, \qquad 0^\sharp \cup 1 = \mho. \tag{5.4}$$

LEMMA 5.2. *For each $s \subset 1_A$, and $\mho = \mho_A{}^A$,*

$$1 \cap s\mho = s, \qquad 0 \cup s\mho = s\mho, \qquad (1 \cup s0^\sharp)0 = s\mho. \tag{5.5}$$

Proof: By (II-c), $1 \cap s\mho \subset s^\sharp s = s$. Conversely, $1 \supset s$ and $s\mho \supset ss = s$, so $1 \cap s\mho \supset s$. Since $0 = \Omega\mho \subset s\mho$, the second equation follows. Next note that $D(s0^\sharp) = (s0^\sharp)^\sharp s0^\sharp \cap 1 = 0ss0^\sharp \cap 1 = 0s0^\sharp \cap 1 = \Omega \cap 1 = \Omega$ by (5.3). This allows us to apply the distributive law (II-b) to get $(1 \cup s0^\sharp)0 = 0 \cup s0^\sharp 0 = 0 \cup s\mho = s\mho$, as asserted.

With these preparations we can formulate

THEOREM 5.3. *For each object A there is a lattice isomorphism ϕ between the lattice of all $s \subset 1_A$ and that of all $q \supset 1_A$, given ($\phi^{-1} = \psi$) by*

$$\phi(s) = 1 \cup s0^\sharp \qquad \psi q = 1 \cap q0.$$

Moreover, $\phi(\Omega) = 1$, $\phi(1) = \mho$, and $\phi(st) = (\phi s)(\phi t)$.

In the model one may readily observe that ϕ carries a subobject, represented by its symmetric idempotent, into the representation of the corresponding quotient object.

Proof: For each s, by Lemma 5.2,

$$\psi\phi(s) = 1 \cap (1 \cup s0^\sharp)0 = 1 \cap s\mathfrak{V} = s.$$

Dually, $\phi\psi$ is the identity. Also $\phi(\Omega) = 1 \cup \Omega 0^\sharp = 1 \cup \Omega = 1$. Clearly, ϕ preserves the partial order. By Theorem 3.3, $\phi(st) = \phi(s \cap t) = (\phi s) \cap (\phi t) = (\phi s)(\phi t)$.

Hitherto, we have represented the kernel of f as the "quotient" $K'f = f^\sharp f \cup 1$. Now we can introduce the kernel in its ordinary meaning as a submodule by

$$\text{Ker } f = \psi(f^\sharp f \cup 1) = 1 \cap (f^\sharp f \cup 1)0. \tag{5.6}$$

Another formula, suggested by the model, is

THEOREM 5.4. *Ker* $f = 1 \cap f^\sharp \Omega f$.

Proof: We first note that, for any $f \in \text{Rel}(A, B)$,

$$\Omega_A{}^B f = (f^\sharp f \cup 1) \cap 0_A{}^A. \tag{5.7}$$

For, by Lemma 5.1, $(f^\sharp f \cup 1) \cap 0 = \Omega(f^\sharp f \cap 1) = \Omega\Omega(f^\sharp f \cup 1) \subset \Omega f(f^\sharp f \cup 1) = \Omega f$, by (4.2). Conversely, $0 = \Omega\mathfrak{V} \supset \Omega f$ and $f^\sharp f \cup 1 \supset f^\sharp f \supset \Omega f$.

This equation, with Theorem 4.7 and equation (5.7), now gives

$$\begin{aligned} \text{Ker } f &= 1 \cap (f^\sharp f \cup 1)0 = 1 \cap (f^\sharp f \cup 1)^\sharp 0 \\ &= 1 \cap [(f^\sharp f \cup 1) \cap 0]^\sharp [(f^\sharp f \cup 1) \cap 0] \\ &= 1 \cap (\Omega f)^\sharp (\Omega f) = 1 \cap f^\sharp \Omega^\sharp \Omega f = 1 \cap f^\sharp \Omega f. \end{aligned}$$

COROLLARY 5.5 *Ker* $f \subset$ *Def* f.

For $f^\sharp \Omega f \subset f^\sharp 1 f = f^\sharp f$, and $Df = \text{Def } f = 1 \cap f^\sharp f$.

Using Ω we can characterize the symmetric idempotents contained in 1 by

THEOREM 5.6. *A symmetric idempotent* u *has* $u \subset 1$ *if and only if* $u\,\Omega = \Omega$.

Proof: If $u \subset 1$, $u\Omega \subset 1\Omega = \Omega$. Conversely, if $u\,\Omega = \Omega$, then by (II-c)

$$u = u^\sharp u \subset u^\sharp \Omega \cup 1 = u\Omega \cup 1 = \Omega \cup 1 = 1,$$

The developments above indicate that the axioms I, II, III for a partially ordered category suffice to prove a number of basic properties valid for additive relations, and suggest the possibility of a representation[4] for such a category by categories of additive relations.

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*REMARKS ON SPECTRA OF OPERATORS ON A LOCALLY CONVEX SPACE**

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The spectra of operators on a locally convex space, defined in the classical way fail to have the useful properties ((*b*)–(*d*) in paragraph 1) enjoyed by the spectra of operators on a Banach space. (See ref. 2.) Waelbroeck[7] introduced a new definition of spectra to maintain these properties. The purpose of this note is to show that these two kinds of spectra coincide for elements of a certain class of operators on a locally convex space.

For the basic theory of locally convex spaces, one may refer to Bourbaki[1] and Grothendieck.[3]

1. *Spectra of Operators.*—Let E be a separated, locally convex space over the complex field C and suppose it is quasi-complete;† let E' be its dual space; let $\mathfrak{L}(E)$ be the algebra of all linear continuous mappings of E into itself. The topology of bounded convergence makes $\mathfrak{L}(E)$ a locally convex algebra. Let $\hat{C}$ be the Riemann sphere, i.e., the one-point compactification of C. If σ is a subset of C, $\bar{\sigma}$ denotes the closure of σ in C and if σ is a subset of $\hat{C}$, $\bar{\sigma}^*$ denotes the closure of σ in $\hat{C}$.

We give the following two definitions of spectra of operators in $\mathfrak{L}(E)$‡—the one in the classical sense (which will be denoted by $sp_c(T)$) and the other given by L. Waelbroeck[7] (which will be denoted by $sp(T)$):

Definition 1: Let $T \in \mathfrak{L}(E)$.

(*a*) $\rho_c(T)$ is defined to be the set of all $\lambda \in C$ such that $\lambda I - T$ is invertible in $\mathfrak{L}(E)$, i.e., all $\lambda \in C$ such that $R_\lambda = (\lambda I - T)^{-1}$ exists in the algebra $\mathfrak{L}(E)$.

(*b*) $\rho(T)$ is defined to be the set of all $\lambda \in \hat{C}$ satisfying the following: there is a neighborhood V_λ of λ in $\hat{C}$ such that for any $\mu \in V_\lambda - \{\infty\}$, $\mu I - T$ is invertible in $\mathfrak{L}(E)$ and the set $\{R_\mu = (\mu I - T)^{-1} / \mu \in V_\lambda - \{\infty\}\}$ is bounded in $\mathfrak{L}(E)$.

(*c*) The set $sp_c(T) = C - \rho_c(T)$ is the *spectrum of T in the classical sense* and $sp(T) = \hat{C} - \rho(T)$ is called the *spectrum* of T.

Obviously, $sp_c(T) \subseteq \overline{sp_c(T)}^* \subseteq sp(T)$.

We can easily see the following properties:

(*a*) If E is a Banach space, then $sp(T) = sp_c(T)$ for all $T \in \mathfrak{L}(E)$. (See, for example, ref. 2, VII, 3.1 and 3.2.)

(*b*) $\rho(T)$ is open in C, hence $sp(T)$ is closed in C.

(*c*) R_λ is analytic§ in $\rho(T) - \{\infty\}$.

(*d*) $sp(T)$ is not empty. (For (*b*)–(*d*), see ref. 7).

If E is nonnormable, then there are operators T for which $sp(T) \neq sp_c(T)$, $\rho_c(T)$ is not open, or $sp_c(T)$ is empty. The following examples show that those cases occur even when E is a Fréchet space.

Example 1: Let $E = C^\omega$ = the product of a countable number of copies of the complex number field, with the product topology. This topology is defined by a countable number of seminorms p_i, $i = 1,2, \ldots$: $p_i(x) = \sup_{1 \leq k \leq i} |x_k|$, where $x = (x_k)_{k=1, 2, \ldots}$, $x_k \in C$. Let $\Lambda = \{\lambda_1, \lambda_2, \ldots\}$ be a sequence of complex numbers.

Then $T_A x = (\lambda_k x_k)_{k=1,2,\ldots}$ for $x = (x_k)_{k=1,2,\ldots}$ defines an element $T_A \in \mathfrak{L}(E)$. Then $sp_c(T_A) = \{\lambda_1, \lambda_2, \ldots\}$ (as a set) and $sp(T_A) = \overline{sp_c(T_A)}^*$. Therefore, if $\{\lambda_i\}$ has a limit point which does not appear in the sequence, then $sp_c(T_A) \neq sp(T_A)$.

Example 2: Let $E = \{f \in C^\infty[0,1] \mid f^{(k)}(0) = 0, \quad k = 0,1,\ldots\}$. Suppose that the topology of E is defined by a countable number of norms p_i, $i = 1, 2, \ldots$:

$$p_i(f) = \sup_{0 \leq k \leq i,\ x \in [0,1]} |f^{(k)}(x)|.$$

Then, the operator T_D: $T_D f = f'$ is continuous, i.e., $T_D \in \mathfrak{L}(E)$. By an elementary calculation, we see that $\lambda I - T_D$ is always invertible in $\mathfrak{L}(E)$ and, in fact, R_λ is given by

$$(R_\lambda f)(x) = e^{\lambda x}\left\{\int_0^x e^{-\lambda t} f(t)\, dt\right\}.$$

From this formula, we see that any complex number λ is in $\rho(T_D)$. Therefore, $\overline{sp_c(T_D)} = \phi$. Since $sp(T_D) \neq \phi$, $sp(T_D) = \{\infty\}$. Hence, in this example, $\overline{sp_c(T_D)}^* \neq sp(T_D)$.

It follows from the above arguments that $sp(T_D{}^{-1}) = \{0\}$. This shows that even though $(T_D{}^{-1})^{-1} = T_D$ exists, $0 \notin \rho(T_D{}^{-1})$.

2. *Completely Bounded Operators.—Definition 2:* $T \in \mathfrak{L}(E)$ is called *completely bounded*†† if there is a neighborhood V of 0 in E such that the image $T(V)$ is bounded in E. Let $\mathfrak{B}(E)$ be the set of all completely bounded operators in E.

It is obvious that if E is a Banach space then any element of $\mathfrak{L}(E)$ is completely bounded, i.e., a bounded operator on a Banach space is completely bounded.†† If E is nonnormable, then the identity $I \in \mathfrak{L}(E)$ is *not* completely bounded, since, otherwise, E has a bounded neighborhood.

A linear operator T on E is completely bounded if and only if there is a continuous seminorm p_o on E such that, for any continuous seminorm q on E, there is a constant $C_q > 0$ satisfying

$$q(Tx) \leq C_q p_o(x) \tag{1}$$

for all $x \in E$ (ref. 6, 2.1).

Definition 3: Let $\mathfrak{A}(E)$ be the subalgebra of $\mathfrak{L}(E)$ generated by $\mathfrak{B}(E)$ and the identity I.

The main result of this note is that $sp(T) = sp_c(T)$ for $T \in \mathfrak{A}(E)$, i.e.,

THEOREM 1. *If $T \in \mathfrak{A}(E)$, then the two kinds of spectra of T, $sp(T)$ and $sp_c(T)$, coincide and are compact in C.*

Since an arbitrary element of $\mathfrak{A}(E)$ can be written in the form $T + \alpha I$ with $T \in \mathfrak{B}(E)$ and $\alpha \in C$, it is enough to prove the theorem for $T \in \mathfrak{B}(E)$.

We might show this in the method which A. Grothendieck used to prove Proposition 5 of chapter V, paragraph 2, 4 and its corollary in reference 3. There, the theorem is reduced to the corresponding theorem on a Banach space where it is known to be true. In this note, we shall give a proof which can be applied exclusively to the theorem on a non-Banach space.

LEMMA 1. *$\mathfrak{B}(E)$ is a two-sided ideal of $\mathfrak{L}(E)$.* This is immediate from the definition.

We remark that $\mathfrak{B}(E) = \mathfrak{L}(E)$ if and only if E is a Banach space.

LEMMA 2. *If $T \in \mathfrak{B}(E)$, so that it satisfies* (1), *then*

(*i*) $R_\lambda = (\lambda I - T)^{-1} = \sum_{n=0}^{\infty} (T^n/\lambda^{n+1})$ *converges and is analytic for* $|\lambda| > r = C_{p_0}$.

(*ii*) $R_\lambda \varepsilon \mathfrak{A}(E)$, *in fact,* $R_\lambda - \lambda^{-1} I \varepsilon \mathfrak{B}(E)$, *if* $|\lambda| > r$.

(*i*) was proved in reference 6, Hifssatz 2.1. From this we know that $sp(T) \subset \{\lambda \mid |\lambda| \leq r\}$. (*ii*) follows immediately from Lemma 1 and the following identity:

$$R_\lambda - \lambda^{-1} I = \lambda^{-1} R_\lambda T.$$

LEMMA 3. *If* $T \varepsilon \mathfrak{B}(E)$ *and if* $\lambda I - T$ $(\lambda \neq 0)$ *has a continuous inverse* R_λ *in* $\mathfrak{L}(E)$ *then* $R_\lambda - \lambda^{-1} I \varepsilon \mathfrak{B}(E)$, *so that* $R_\lambda \varepsilon \mathfrak{A}(E)$.

We can prove this lemma in the same way as Lemma 5.7.1 in reference 4, using the above lemmas.

Proof of the Theorem: From Lemma 2, $sp(T)$ is compact for $T \varepsilon \mathfrak{A}(E)$. Since the theorem is known to be true when E is a Banach space, we suppose that E is non-normable. Then $T^{-1} \epsilon \mathfrak{L}(E)$, so that $0 \varepsilon sp_c(T) \subseteq sp(T)$. Next, let $\lambda \varepsilon \rho_c(T)$, $|\lambda| \leq r$, $\lambda \neq 0$. Then, by Lemma 3, $R_\lambda \varepsilon \mathfrak{A}(E)$. Hence R_λ has a compact spectrum. Then it follows from the Waelbroeck's theorem (ref. 7, p. 4) that $\lambda \varepsilon \rho(T)$. Thus, using Lemma 2 again, we see that $sp_c(T) = sp(T)$.

3. *A Remark on the Spectra of Compact Operators.*—An operator $T \varepsilon \mathfrak{L}(E)$ is called *compact*** if there is a neighborhood V of 0 in E whose image $T(V)$ is relatively compact in E. Let $\mathfrak{K}(E)$ be the set of all compact operators on E. From the definition, it is obvious that a compact operator is completely bounded, i.e., $\mathfrak{K}(E) \subseteq \mathfrak{B}(E)$. Hence, by Theorem 1, $sp_c(T) = sp(T)$ for $T \varepsilon \mathfrak{K}(E)$. Therefore, by the results in Leray[5] or Grothendieck,[3] we have the following:

THEOREM 2. *If* $T \varepsilon \mathfrak{K}(E)$, *then* $sp(T) = sp_c(T)$ *consists of a finite number of points or of 0 and a sequence of points converging to zero. Moreover, every nonzero point in* $sp(T)$ *is an eigenvalue of* T.

Summarizing the whole arguments on spectra of operators, we can form the following table:

E		Classes of Operators				
Finite dim. space	$\longleftrightarrow$	$\mathfrak{K}(E)$	$=$	$\mathfrak{B}(E)$	$=$	$\mathfrak{L}(E)$
Banach space	$\longleftrightarrow$	$\mathfrak{K}(E)$	$\subset$	$\mathfrak{B}(E)$	$=$	$\mathfrak{L}(E)$
Locally convex sp.	$\longrightarrow$	$\mathfrak{K}(E)$	$\subset$	$\mathfrak{B}(E)$	$\subset \mathfrak{A}(E) \subset$	$\mathfrak{L}(E)$
		$\downarrow$			$\downarrow$	
(spectrum $\rightarrow$)		($sp_c = sp$, finite or a seq. of pts. $\rightarrow 0$)			($sp_c = sp$, compact in C)	

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† A space E is quasi-complete if every closed bounded set in E is complete. We remark that every quasi-complete normed space is a Banach space.

‡ We can consider the spectra of any linear transformations T defined on $D_T \subseteq E$ into E. In this case, the definitions of both kinds of spectra will be the same as Definition 1.

§ $T_\lambda \epsilon \mathfrak{L}(E)$ is called analytic in λ on an open set $\Omega \subseteq C$, if the complex-valued functions $f_{x,x'}(\lambda) = \langle T_\lambda x, x' \rangle$ are analytic in Ω for all $x \epsilon E$, $x' \epsilon E'$. See reference 4, chapter III, paragraph 2.

†† Schaefer called it "eine beschränkte Transformation"[6] and Grothendieck, "un opérateur borné."[3] In this connection, we may use the terminology "bounded" instead of "completely

bounded." Here, we put "completely" simply because "a bounded operator" might mean such an operator that maps any bounded set into a bounded set.

** This is an extension of the notion of compact operators on Banach spaces (see ref. 2, chapter VI). Leray used "complètement continu" in reference 5 for compact.

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ON THE WEIGHTED POLYNOMIAL APPROXIMATION IN A LOCALLY COMPACT SPACE

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Let E be a locally compact space, $C(E)$ the algebra of all continuous real functions on E, and $C_\infty(E)$ the Banach space of all continuous real functions on E vanishing at infinity endowed with its natural norm $f \to \|f\| = \sup\{|f(x)|; x \in E\}$ for $f \in C_\infty(E)$. Let us consider a subalgebra $\mathfrak{A}$ of $C(E)$ containing 1, and a vector subspace $\mathfrak{W}$ of $C_\infty(E)$ which is an $\mathfrak{A}$-module, that is, $\mathfrak{A}\mathfrak{W} \subset \mathfrak{W}$. The general Bernstein approximation problem on a locally compact space E consists in asking for a description of the closure of such a $\mathfrak{W}$ in $C_\infty(E)$. We look for a general treatment similar to the one devised by Stone in the case of the Weierstrass approximation theorem.[1] If we define $x' \sim x''$ provided $x', x'' \in E$ and $f(x') = f(x'')$ for any $f \in \mathfrak{A}$, we obtain an equivalence relation induced on E by $\mathfrak{A}$, to be denoted $E/\mathfrak{A}$. Every equivalence class X of E modulo $\mathfrak{A}$ is closed in E, hence locally compact. We may consider the vector subspace $\mathfrak{W}|X$ of the Banach space $C_\infty(X)$, formed by the restrictions to X of all members of $\mathfrak{W}$. We shall say that $\mathfrak{W}$ is of *finite type* under $\mathfrak{A}$ if the following condition is fulfilled: $f \in C_\infty(E)$ belongs to the closure of $\mathfrak{W}$ in $C_\infty(E)$ if (and only if) $f|X \in C_\infty(X)$ belongs to the closure of $\mathfrak{W}|X$ in $C_\infty(X)$ for every equivalence class X of E modulo $\mathfrak{A}$. If $\mathfrak{W}$ is of finite type under $\mathfrak{A}$ and $\mathfrak{A}$ is separating, that is, if $E/\mathfrak{A}$ is the identity, then $\mathfrak{W}$ is dense in $C_\infty(E)$ if and only if, for every $x \in E$, there is some $w \in \mathfrak{W}$ such that $w(x) \neq 0$. In case $\mathfrak{W}$, as an $\mathfrak{A}$-module, has one generator w, that is, $\mathfrak{W} = \mathfrak{A}w$, then for $\mathfrak{W}$ to be dense in $C_\infty(E)$ it is necessary that $\mathfrak{A}$ be separating and that w never vanish, these two conditions being also sufficient in case $\mathfrak{W}$ is of finite type under $\mathfrak{A}$. When $\mathfrak{A} = \mathfrak{W}$, we are reduced to the situation in the Weierstrass-Stone theorem. When $\mathfrak{A} = R$ is reduced to the constant functions, $\mathfrak{W}$ is the most general vector subspace of $C_\infty(E)$,

and the general case is to be reduced to this particular case combined with the Hahn-Banach theorem.

THEOREM 1. *If all functions in $\mathfrak{W}$ have compact supports (in particular, if E is compact), then $\mathfrak{W}$ is of finite type under $\mathfrak{A}$.*

If E is compact and $\mathfrak{A} = \mathfrak{W}$, this theorem gives us as a particular case the Weierstrass-Stone theorem. On the other hand, Theorem 1 follows from the Weierstrass-Stone theorem if we want to prove it directly; or it is a consequence of Theorem 2 (more precisely, of Corollary 1 in its exponential form) below. As Theorem 1 points out, a suitable rate of decrease at infinity of the functions in $\mathfrak{W}$ with respect to the functions in $\mathfrak{A}$ should be sufficient to ensure the finite type case.

To proceed to more precise conditions in such a direction, let us introduce the following notation. Call $\mathcal{P}_n$ $(n = 1, 2, \ldots)$ the algebra of all real polynomials on Euclidean real n-space R^n and let $\mathfrak{B}_n$ $(n = 1, 2, \ldots)$ be the set of all continuous, everywhere strictly positive real functions B on R^n that are rapidly decreasing at infinity, that is, $\mathcal{P}_n B \subset C_\infty(R^n)$, and that are fundamental weight functions in the sense of the classical Bernstein approximation problem, that is, $\mathcal{P}_n B$ is dense in $C_\infty(R^n)$. Let A be a set of generators for the algebra $\mathfrak{A}$, so that every function in $\mathfrak{A}$ is a real polynomial in a finite number of functions in A. Let also W be a set of generators for $\mathfrak{W}$ as an $\mathfrak{A}$-module, so that every function in $\mathfrak{W}$ is a finite linear combination of functions in W with coefficients in $\mathfrak{A}$.

THEOREM 2. *If corresponding to any generators $f_1, \ldots, f_n \in A$, $w \in W$, there is some $B \in \mathfrak{B}_n$ such that*

$$|w(x)| \leqq B[f_1(x), \ldots, f_n(x)] \quad \text{for } x \in E,$$

then $\mathfrak{W}$ is of finite type under $\mathfrak{A}$.

This theorem reduces the search of sufficient conditions for the finite type case in the general Bernstein approximation problem to the search of sufficient conditions for fundamental weight functions in the finite-dimensional classical Bernstein approximation problem. The closure problem in the general case is then reduced to the density problem in the finite-dimensional case. Although this theorem has only the nature of a sufficient condition, actually it has the following converse. Let $\mathfrak{F}_n$ $(n = 1, 2, \ldots)$ be a set of continuous, everywhere strictly positive real functions on R^n. Then Theorem 2 is true for any E, $\mathfrak{A}$, $\mathfrak{W}$, A, W if we replace $\mathfrak{B}_n$ by a given $\mathfrak{F}_n$ if and only if $\mathfrak{F}_n \subset \mathfrak{B}_n$ $(n = 1, 2, \ldots)$, so that Theorem 2 is the best possible in a sense.

We now turn to a reduction of the general closure problem to the density problem in the one-dimensional case. Let $\mathfrak{F}$ be a set of continuous, everywhere strictly positive real functions on the real line R such that, if $B_1, \ldots, B_n \in \mathfrak{F}$ and B is defined on R^n by $B(t_1, \ldots, t_n) = \inf\{B_1(t_1), \ldots, B_n(t_n)\}$, then $B \in \mathfrak{B}_n$ (so that, in particular, $\mathfrak{F} \subset \mathfrak{B}_1$).

THEOREM 3. *If corresponding to any generators $f \in A$, $w \in W$, there is some $B \in \mathfrak{F}$ such that*

$$|w(x)| \leqq B[f(x)] \quad \text{for } x \in E,$$

then $\mathfrak{W}$ is of finite type under $\mathfrak{A}$.

Again, in spite of the fact that this result has only the character of a sufficient condition, it holds true for any E, $\mathfrak{A}$, $\mathfrak{W}$, A, W, and a given $\mathfrak{F}$ if and only if $\mathfrak{F}$ has the property stated immediately before the theorem.

Let $\mathfrak{B}_n$ ($n = 1, 2, \ldots$) be the subset of $\mathcal{B}_n$ of all continuous, everywhere strictly positive real functions B on R^n such that $B^a \in \mathcal{B}_n$ for all real exponents $a > 0$.

THEOREM 4. *If corresponding to any generators $f \in A$, $w \in W$, there is some $B \in \mathfrak{B}_1$ such that*

$$|w(x)| \leqq B[f(x)] \quad \text{for } x \in E,$$

then $\mathfrak{W}$ is of finite type under $\mathfrak{A}$.

COROLLARY 1. *If corresponding to any generators $f \in A$, $w \in W$, there is an even, continuous, everywhere strictly positive real function B on R such that*

$$|w(x)| \leqq B[f(x)] \quad \text{for } x \in E,$$
$$\log 1/B(t) \text{ is a convex function of } \log t \text{ for } t > 0,$$
$$\int^{+\infty} 1/t^2 \cdot \log 1/B(t) \cdot dt = +\infty,$$

(in particular, if corresponding to any generators $f \in A$, $w \in W$, there are real constants K, $k > 0$ such that

$$|w(x)| \leqq K \cdot \exp[-k|f(x)|] \quad \text{for } x \in E),$$

then $\mathfrak{W}$ is of finite type under $\mathfrak{A}$.

COROLLARY 2. *If, for any generators $f \in A$, $w \in W$, we have*

$$\sum_{n=1}^{+\infty} 1/M_n^{1/n} = +\infty, \qquad \text{where} \quad M_n = \|f^n w\| \quad (n = 0, 1, \ldots),$$

then $\mathfrak{W}$ is of finite type under $\mathfrak{A}$.

For instance, if W is reduced to one function w, that is $\mathfrak{W}$ has one generator as an $\mathfrak{A}$-module and the above series diverges for every $f \in A$, then $\mathfrak{W}$ is dense in $C_\infty(E)$ if and only if $\mathfrak{A}$ is separating and w never vanishes (Malliavin).

The above results in the case of a separating algebra $\mathfrak{A}$ imply the same results in the general case provided we deal from the very start with vector valued functions and continuous sums of normed spaces in the sense of von Neumann. This leads also, more generally, to statements of the general Bernstein approximation problem and the preceding theorems and corollaries in terms of commutative algebras of operators in real normed spaces and a suitable notion of generalized local convexity.[2] Extension to commutative self-adjoint algebras of operators in complex normed spaces is immediate. Extension to the general complex case is not yet possible at this moment, since even the commutative (not necessarily self-adjoint) complex case on compact spaces (Wermer, etc.) and the self-adjoint (not necessarily commutative) complex case in Hilbert spaces (Kadison, Glimm, etc.) are not yet completely clarified from the viewpoint of the Weierstrass approximation and *a fortiori* from the viewpoint of the Bernstein approximation.

[1] Stone, M. H., "The generalized Weierstrass approximation theorem," *Mathematics Magazine*, **21** (1948); Malliavin, P., "L'approximation polynomiale ponderée sur un espace localement compact," *American Journal of Mathematics*, **81** (1959). An excellent report on the classical Bernstein approximation problem is due to S. N. Mergelyan, "Weighted approximations by polynomials," *Uspehi Matematičeskih Nauk* (n.s.), vol. 11 (1956), *American Mathematical Society Translations* (Series 2), **10** (1958).

[2] See Nachbin, L., "Algebras of finite differential order and the operational calculus," *Ann. Math.*, **70** (1959).

*THE PATTERN OF CONGENITAL TRANSMISSION OF AN AVIAN LEUKOSIS VIRUS**

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It was recently reported that cells derived from some chick embryos are highly resistant to infection with Rous sarcoma virus (RSV) when challenged *in vitro*.[1] A virus was isolated from the resistant cells which induced resistance to RSV in chick embryo cells. Although the virus was named RIF, an acronym for "resistance inducing factor," it proved to be indistinguishable from the virus of visceral lymphomatosis (VLV) in its physicochemical, immunological, and biological characteristics.[1, 2] It has been shown that four established strains of VLV can be detected in tissue culture by interference with RSV,[1] and we recently observed that avian myeloblastosis virus can also be detected in this way.

The epidemiological behavior of RIF further supported its suggested relation to VLV. RIF, for example, was found to occur in highest frequency in the embryos of a chicken flock selected for a high incidence of neural and visceral lymphomatosis. The occurrence of RIF in embryos apparently normal in every respect indicates that congenital infection plays an important role in perpetuating the virus under natural conditions. A similar picture for VLV has been reported by Burmester[3] who found that VLV could be isolated from apparently healthy embryos. Recently we have isolated RIF from 22 of 33 cases of lymphomatosis occurring in the field in Northern California. Since RIF and VLV have given identical results in every characteristic which has been examined, RIF should be considered a California strain of VLV.

The *in vivo* techniques which have been used to assay VLV are extremely cumbersome and time-consuming.[4] By way of comparison the assay of RIF and various other VLV strains by interference with RSV is quick and efficient. The utility of the assay and the proximity of a heavily infected flock of chickens facilitated the present detailed investigation of the congenital transmission of RIF. The roles of male and female parents in congenital infection and particularly the relation of parental viremia and antibody titer to congenital infection were evaluated. The evidence to be presented shows that the parental flock was composed of birds with and without persistent RIF-viremia. Those with viremia had no detectable neutralizing antibody to RIF. Those without viremia had neutralizing antibody, and the titer of antibody remained relatively constant over a 10-month period.

All the viremic hens transmitted infection through the egg to most of their progeny while only one of the nonviremic hens did so. Viremic roosters failed to transmit infection congenitally.

Materials and Methods.—Source and preparation of cultures for assay of RIF and RSV: Ten-day embryos were obtained from a commercial strain of white leghorn chickens with a relatively low incidence (about 1 in 30) of RIF-infected embryos. Each embryo was trypsinized individually and several cultures were made from each embryo according to the procedure previously described.[1] Four of the cultures from each embryo were infected immediately with two dilutions of Rous sarcoma virus (RSV) to determine whether they were congenitally infected with RIF. Only

those embryos which were highly sensitive to RSV infection were subsequently used for assay purposes.

Source of birds for the congenital transmission studies: Kimber Farms strain 13 is an experimental flock which has been selected for over ten years for a high incidence of lymphomatosis by

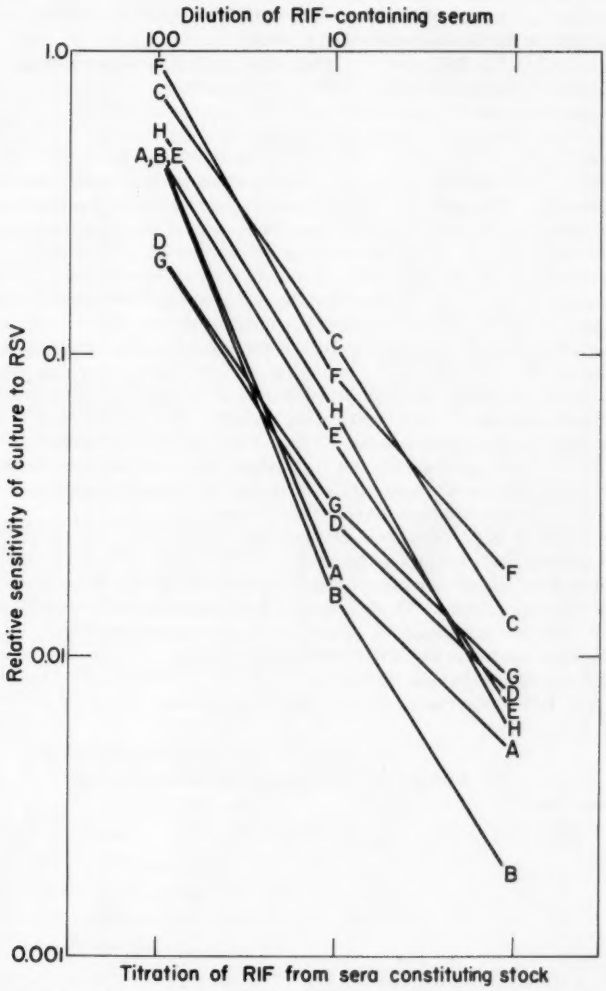

FIG. 1.—Titration of RIF from sera constituting stock. 0.1 ml of the appropriate dilution of each serum was added to RSV-sensitive cultures which were transferred and challenged with RSV at 5 days. The letters A–H refer to the individual viremic birds.

mating siblings of chickens which have developed either neural or visceral lymphomatosis. To obtain embryos of known parentage, each male was placed in a separate pen with a group of females and fertile eggs were obtained from the females by trap-nesting. In the present series of experiments, there were 18 hens of which 15 were fertile, and 8 roosters. These were part of a larger study employing some 75 hens and ten roosters, and it will be reported at a later date. The

parental birds averaged about 12 months of age at the beginning of the laying period, which lasted about a month and a half. At the last of four bleedings, the parental birds were about 22 months old.

Rous sarcoma virus stock and assay: The high titer stock of Bryan was used in the present experiments.[1] The immediate source was a pool of homogenized chicken tumors which had about 10^7 focus-forming units (FFU) per ml. The virus was assayed by focus formation on chick embryo cells *in vitro* by the technique described in 1960.[1]

RIF stock and assay: The RIF stock was obtained by pooling the plasmas of eight congenitally infected birds and centrifuging the pool at $5000 \times g$ for 10 minutes. The supernate was frozen at −90°C. The virus was assayed by adding 0.1 ml of the appropriate dilution to two chick embryo cultures as previously described.[1] These were overlaid with agar the next day and incubated for five days before transfer to six plates. Four of the cultures were challenged with appropriate dilutions of RSV. The remaining two cultures were used for further transfer and assay at three- to four-day intervals. The results of assay of each individual serum constituting the pool are presented in Figure 1. The titer of the pool was the average of the constituent individuals. It can be seen that there was an almost linear inverse relationship at the first transfer between the concentration of RIF and the number of RSV foci up to the RIF concentration which reduced the RSV foci by a factor of about 50. Further transfer and challenge of higher dilutions of the pool showed that the stock contained about 10^7 infective units of RIF per ml.

Assay of antibodies to RSV: Antisera to RSV neutralize RIF and antisera to RIF neutralize RSV.[1] Because of this immunological relationship, and the ease of carrying out quantitative neutralization studies with RSV, the RSV neutralization served as a useful, but not infallible (see Results) gauge of antibodies to RIF. Sera were heated at 56°C for 30 minutes before use in neutralization tests. Approximately 5,000 FFU of RSV in 1.0 ml of complete medium[1] were incubated at 37°C for 40 minutes with the appropriate dilution of serum. At the end of this period, 0.1 ml of the mixture was assayed for surviving RSV infective particles. Antiserum to RSV was obtained from hyperimmunized convalescent chickens.

Assay of antibodies to RIF: The assay of antibodies to RIF was more complicated than the assay for antibodies to RSV, and lacked the precision of the latter. A 1:10 dilution of RIF stock was incubated at 37°C for 40 minutes with the appropriate dilution of serum and 0.1 ml was plated on RSV sensitive cultures. These were transferred and challenged with RSV at five days. The number of RSV foci was compared to the number in a control to which no RIF had been added, and to another control in which RIF alone had been added. Neutralization was considered positive if the serum eliminated the RSV-inhibiting effect of the RIF, i.e., if the culture treated with the serum + RIF mixture gave as many foci upon challenge with RSV as did the RIF-free control.

TABLE 1

RIF-VIREMIA AND ANTIBODY IN PARENTAL BIRDS

Viremic Birds[a]			Nonviremic Birds[b]					
	Antibody to			Antibody to			Antibody to	
Hen No.	RSV[c]	RIF[d]	Hen No.	RSV[c]	RIF[d]	Hen No.	RSV[c]	RIF[d]
1	−	−	2	−	+	16	−	+
3	−	−	4	+	+	18	+	+
7	−	−	5	+	+	19	+	+
9	−	−	8	−	+	20	+	+
Rooster No.						Rooster No.		
R1	+	−	10	+	+	R3	+	+
R2	−	−	11	+	+	R4	+	+
R9	−	−	12	+	+	R6	+	+
			13	+	+	R8	+	+
			14	−	+	R10	+	+

[a] Viremic birds = Serum obtained at 12, 14, 17, and 22 months of age and added to RSV-sensitive cultures induced resistance to RSV at the first transfer.

[b] Nonviremic birds = Serum failed to induce resistance in cultures challenged with RSV in three successive transfers.

[c] Antibody to RSV: The (+) = a 1:10 dilution or serum reduced RSV titer ≥ 10-fold in 40 minutes of incubation at 37°C. The (−) = a 1:10 dilution of serum reduced RSV titer ≤ 2-fold in 40 minutes of incubation at 37°C.

[d] Antibody to RIF: The (+) = a 1:10 dilution of serum eliminated the RSV-inhibitory effect of a 1:10 dilution of RIF in cultures challenged with RSV after 1 transfer. The (−) = a 1:10 dilution of serum failed to eliminate the RSV-inhibitory effect of RIF.

Results.—RIF and antibody in parental sera: The levels of RIF and neutralizing antibodies in the sera of each of the parental birds were assayed during the egg-laying period and at three post-laying intervals up to ten months after the first bleeding. Since there was no marked change in the concentration of either virus or antibody of any individual bird during this period, they are simply classified as positives and negatives according to virus and antibody status in Table 1. RIF was found in high concentration in the sera of four of the 18 hens and three of the eight roosters. None of the remaining birds had detectable viremia at any time during the experiment.

The immunologic specificity of the RIF obtained from each bird was determed by incubation with antiserum to RSV. In every case the virus could be neutralized by the anti-RSV serum, indicating the antigenic similarity of the virus from each bird.

Only one of the eight viremic birds, rooster R1, had detectable neutralizing antibody to RSV and none had antibody to RIF. In marked contrast to the absence of antibody in the viremic group, 14 of the 18 nonviremic birds had antibody to RSV and all had antibody to RIF.

Except for rooster R1, each of the viremic birds had a high enough concentration of RIF to induce at least a 10-fold increase in resistance to RSV after only a single transfer of RSV-sensitive cells indicating a substantial virus titer.[1] (Rooster R1, which was also the only viremic bird with antibodies to RSV, had five to ten times less virus in the serum than did the others). An experiment was carried out to determine more precisely the virus titer in the sera of the viremic hens. In determining the titer, advantage was taken of the approximately linear inverse relationship that exists between RIF concentration in the original inoculum used to infect chick embryo cultures and the number of foci which results from challenge with RSV after one transfer. The various sera and a stock suspension of RIF were decimally

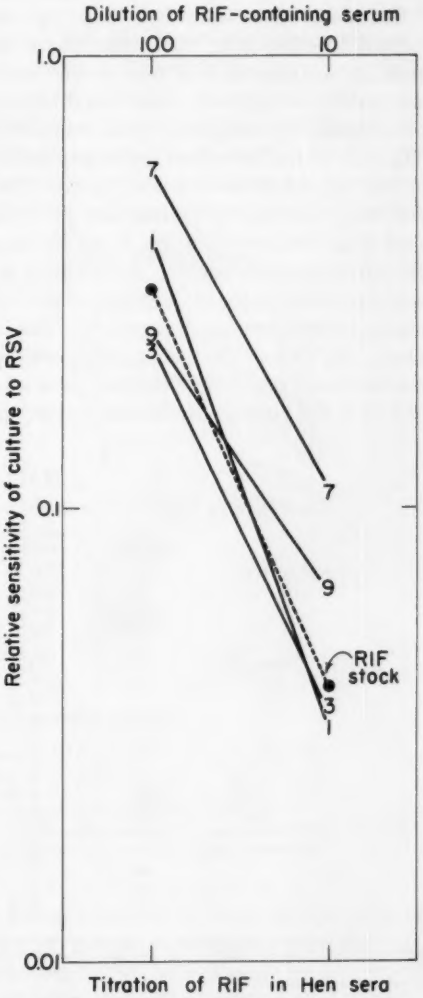

FIG. 2.—Titration of RIF in hen sera. See legend for Fig. 1. The numbers refer to the viremic hens.

diluted and 0.1 ml of each added to sensitive cultures. The infected cultures were transferred at five days and challenged with RSV. The relative sensitivities of the cultures are presented in Figure 2. The results show that the sera of three of the viremic hens had about the same concentration of RIF as the stock virus, which was itself a pool of sera obtained from eight other viremic birds. The serum of hen No. 7 had about three times less virus than the others. When these results are considered together with the assays of the sera from the eight stock virus birds, a striking similarity in RIF titer is seen among the sera of all viremic birds without antibody. An approximation could be made of the number of infectious units per ml of serum by comparing these results with a similar titration of the stock virus. The titer of the latter had previously been determined as about 10^7 infectious units of RIF per ml by an end-point titration[1] after four successive transfers of infected cultures; since the individual sera gave results similar to the stock in the abbreviated single transfer titration, it can be assumed they also contained approximately 10^7 infectious units per ml. As the virus is thermolabile,[2] virus production must be continuous and rapid in order to maintain the high levels found in the blood. Even higher concentrations of virus were found in extracts of various organs of viremic birds. In view of the findings discussed below on the plating of infected cells from rooster testes and from embryos, these observations suggest that high proportions of cells in the viremic adults were actively producing virus.

TABLE 2

COMPARATIVE TITRATION OF ANTIBODIES TO RSV AND RIF IN HEN SERA

	Hen No.	Antibody to RSV		Antibody to RIF	
		12 mos.	17 mos.	12 mos.	17 mos.
Viremic Hens	1	<0.5[a]	<0.5	N.T.[b]	<1
	3	<0.5	<0.5	N.T.	<1
	7	<0.5	<0.5	N.T.	<1
	9	<0.5	<0.5	N.T.	<1
Nonviremic Hens	2	<0.5	<0.5	N.T.	1–2
	4	1.5	1.3	1–2	1–2
	5	2.6	2.3	2–3	2–3
	8	<0.5	<0.5	1–2	1–2
	10	2.7	N.T.	>3	N.T.
	11	1.8	1.6	2–3	N.T.
	12	2.7	N.T.	2–3	N.T.
	13	1.8	1.3	>2	>2
	14	<0.5	<0.5	1–2	1–2
	16	N.T.	<0.5	N.T.	1–2
	17	3.5	3.0	>3	4–5
	18	2.7	2.0	>3	2–3
	19	2.7	2.0	>3	2–3
	20	1.9	1.6	>2	2–3

[a] Anti-RSV titer is expressed as the $\log_{10}$ of the serum dilution which inactivated 90 per cent of a suspension of RSV containing about 5,000 F.F.U. per ml. The value was determined by plotting $\log_{10}$ serum dilution vs $\log_{10}$ RSV survivors and extrapolating the linear portions of the curves of each serum in the region where the curves paralleled one another.

[b] Anti-RIF titer is expressed as the interval between the $\log_{10}$ of the serum dilutions at which there was, successively, evident neutralization and no neutralization of a 1:10 dilution of RIF stock.

N.T. = Not tested in this experiment.

A detailed representation of antibody to RSV and RIF in the individual hen sera at the beginning of the experimental period and five months later is seen in Table 2. The results of Table 2 indicate that the anti-RIF activity of a group of sera can be adequately gauged by anti-RSV activity of the sera. In general it was found that

the viremic sera with no anti-RSV activity had no anti-RIF activity (an exception noted before is viremic serum R1 in Table 1 which had substantial anti-RSV activity and no anti-RIF activity). Among the nonviremic sera, those with little or no anti-RSV activity (Nos. 2, 4, 8, 14, and 16) had low anti-RIF activity while those with high anti-RSV activity had high anti-RIF activity. The coefficient of correlation is about 0.8.

The results in Table 2 also show that no substantial change occurred in the antibody levels of the sera over a five-month interval. The situation was essentially the same at the end of 10 months, when a less extensive test was carried out.

Congenital infection of embryos: RIF infection of embryos was detected by culturing cells of individual embryos of known parentage and challenging the cultures with specified dosages of RSV. In most cases only primary and secondary cultures were tested in this way, although tertiary cultures of some embryos were also tested.

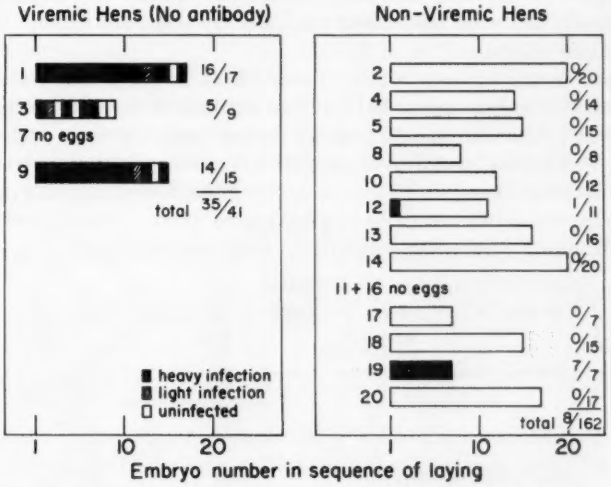

FIG. 3.—Congenital transmission of RIF. Cultures were prepared from trap-nested embryos. Primary, secondary, and in some cases tertiary cultures were challenged with RSV. "Heavy infection" indicates those embryos whose cells were resistant to RSV in primary culture. "Light infection" indicates those sensitive as primary cultures but resistant as secondaries. "Uninfected" indicates those sensitive to RSV through 2 or 3 transfers.

Preliminary work had shown that when a high proportion of cells derived from an embryo had become infected with RIF *in ovo*, the primary cultures from that embryo would be highly resistant to RSV infection. If only a small proportion of cells from the embryo was infected, the *primary* culture would be sensitive to RSV, but the culture would become resistant in subsequent transfers as a higher proportion of the cells became infected.

Infected and noninfected embryos are shown in sequence of laying in Figure 3. It can be seen that the three viremic hens which laid fertile eggs produced a high proportion (86 per cent) of infected embryos. Two of the viremic hens (Nos. 1 and 9) infected all but one of their progeny, while the third (No. 3) infected five

of her progeny of nine. More recent studies with larger numbers of birds show that all viremic hens produce infected progeny.

Only one of the 12 fertile, nonviremic hens (No. 19) regularly had infected progeny. It is evident from the antibody data for this hen in Table 2 that congenital transmission may occur despite the presence of relatively high concentrations of antibody. Thus, it is likely that active multiplication of RIF continues in the intracellular environment of the ova for prolonged periods while these cells are continuously bathed by antibody. Indeed, since it is well established that maternal antibody is passively transferred through the yolk, and the yolk is continuous with the cytoplasm of the ovum[5] it is possible that multiplication of the viral genome proceeds in the presence of *intra* cellular antibody.

Only a single infected embryo among 154 was obtained from the remaining fertile nonviremic hens, i.e., the first embryo derived from hen No. 12. Subsequently this hen produced 10 noninfected embryos. Subsequent studies with larger numbers of birds has shown, however, that about one of every eight nonviremic hens in this flock is a congenital transmitter of RIF.

In contrast to the obvious role of viremic hens in congenital transmission of RIF, the viremic roosters appear to be unable to transmit the virus to their progeny at fertilization (Table 3). All 37 progeny issuing from nonviremic hens, Nos. 17, 18, and 20, which were mated to three different viremic roosters were uninfected. Although nonviremic hen No. 19 was mated to a viremic rooster and produced infected progeny, she continued to do so when mated to a nonviremic rooster indicating that congenital transmission was strictly under maternal control.

TABLE 3

FAILURE OF CONGENITAL TRANSMISSION BY VIREMIC ROOSTERS

	Rooster no. (Mated with)	Hen no.	Infected progeny/ total progeny
Viremic Roosters	R1	18	0/15
	R2	17	0/7
	R9	19*	7/7
	"	20	0/17
		Total	7/46
Nonviremic Roosters	R3	14	0/20
	R4	13	0/16
	R6	4	0/14
	"	5	0/15
	R8	1†	16/17
	"	2	0/20
	"	8	0/8
	"	9†	14/15
	"	10	0/12
	"	12	1/11
	R10	3†	5/9
		Total	36/157

* Continued to produce infected progeny when mated with nonviremic rooster.
† Viremic hens.

Further studies on the failure of male transmission: The failure of viremic roosters to transmit infection to their progeny might have been due to inactivation of virus carried in the sperm by antibody present in the oviduct of fertilized hens. To evaluate the role of maternal antibody in the failure of male transmission, hens Nos. 2 and 8 and the group in Table 1 and another hen, No. 33, all with no antibody to RSV and with low antibody titers to RIF were mated to viremic rooster No. R9. Two embryos from each hen were used to prepare primary cultures and these,

as well as secondary and tertiary cultures were challenged with RSV. All cultures remained fully sensitive to RSV, indicating the embryos were uninfected and that there is no male transmission of RIF even when heavily viremic males are mated to hens with little antibody.

Another possibility which was considered to explain the failure of male transmission was that the testicular cells of viremic roosters were resistant to infection. One of the viremic roosters, R2, was sacrificed and his testes removed. To determine whether or not cells of the testes were infected and to obtain an estimate of the extent of infection, the cells were suspended by trypsinization, washed, counted, and plated on RSV-sensitive chick embryo cells. An aliquot of the testicular cells was disrupted by freezing and thawing, and also plated on sensitive cells. If the cells were actively producing RIF, the intact cells should be more effective than the disrupted cells in inducing a high level of resistance to RSV in the sensitive cultures. If the testicular cells were merely contaminated with RIF, there should be no effect of cell disruption on the speed or extent of the induction of resistance to RSV in sensitive cultures.

The results in Table 4 show that the intact cells were more efficient than the disrupted cells in inducing resistance to RSV. They induced a 50-fold increase in resistance to RSV after a single transfer of the sensitive cultures, while the disrupted cells induced resistance only after two transfers. Reference to standard curves for the titration of RIF[1] indicates that at least 10 times as much infectious

TABLE 4

VIRUS PRODUCTION BY TESTICULAR CELLS OF VIREMIC ROOSTER R2*

	No. cells or cell-equivalents plated	Relative sensitivity at 1st transfer	Relative sensitivity at 2nd transfer
Intact cells	1.6×10^5	0.021	0.002
Disrupted cells	1.6×10^5	1.0	0.03

* Testicular cells were suspended with trypsin and counted. An aliquot was frozen and thawed three times to disrupt cells. The appropriate numbers of intact cells or disrupted cell-equivalents were plated on sensitive cultures which were challenged after one transfer (5 days) and two transfers (5 + 3 days).

virus was produced by the intact cells in the first few days after plating than was physically associated with the cells at the time of their disruption. At least 10^6 infectious units of free virus are required to induce the level of resistance found within a single transfer after the plating of 1.6×10^5 intact cells. The fact that 1.6×10^5 testicular cells released enough virus to induce this level of resistance indicates that a high proportion of the testicular cells were producing virus, and lends all the more significance to the failure of males to congenitally transmit RIF.

The proportion of infected cells in the embryos: It was observed above that cell cultures derived from most congenitally infected embryos were highly resistant to RSV when challenged immediately after explantation, suggesting that most of the cells had become infected *in ovo*. This seemed remarkable in view of the fact that the infected embryos were perfectly developed and indistinguishable from uninfected embryos. Indeed, subsequent work has shown that congenitally infected embryos hatch and mature in a normal manner. It seemed worth while, therefore, to seek stronger support for the suggestion that a high proportion of cells from these embryos was infected. Cell suspensions were prepared from six embryos which were derived from hens known to be congenital transmitters of RIF. Cultures were prepared from a portion of the suspended cells from each embryo and challenged

immediately with RSV. The remaining portion of cells was washed free of extracellular virus and counted. The suspensions were diluted and 50 cells and ten cells from each embryo were plated on sensitive chick cultures. Aliquots of these cultures were challenged with RSV after three transfers. If a relatively high proportion of cells was infected, it was anticipated from the standard curves referred to above[1] that the plating of ten virus producing cells would induce resistance within three transfers.

TABLE 5

PROPORTION OF INFECTED CELLS IN CONGENITALLY INFECTED EMBRYOS

	Hen No.	Embryo	Relative sensitivity of primary cultures to direct challenge with RSV	Plating of cells on Sensitive cultures	
				50 cells	10 cells
Experiment A	1	a	0.009	0.004*	0.04*
		b	0.04	N.T.	0.58
		c	0.07	0.03	0.06
		d	0.10	0.35	0.94
		e	0.05	0.04	0.4
		f	0.03	0.01	0.26
		Control	1.0	1.0	
				10 cells	2 cells
Experiment B	60	a	0.0002	0.003†	0.024†
		b	0.008	0.001	0.004
		c	0.007	0.017	0.030
		Control	1.0	1.0	

* Relative sensitivity of sensitive cultures which had been mixed with the indicated number of cells from infected embryos, transferred three times and challenged with RSV.
† As above, but challenged after four transfers.
See text for procedure.

The results of this experiment are presented in Table 5A. The results show that those embryos which were the source of highly resistant primary cultures were comprised of a high proportion of cells actively producing virus, since the plating of ten cells on sensitive cultures induced resistance within three transfers.

Another experiment was carried out in which only ten and two cells from three congenitally infected embryos were plated on sensitive cultures. The embryos were obtained from a nonviremic hen (No. 60) with high antibody titer. This hen was not included in the organical group, but was found to transmit infection to almost all of her progeny. The cultures were transferred four times and challenged with RSV. The results in Table 5B show that even two cells from a RIF infected embryo which was highly resistant to RSV in primary culture could induce resistance to RSV in a sensitive culture. This was unmistakable evidence that a high proportion of cells in highly resistant embryos were infected with RIF.

It should be emphasized, however, that not all RIF-infected 10-day-old embryos have such a high proportion of their cells infected. In Figure 3 it can be seen that the resistance of some infected embryos can be detected only in the second transfer of cultures made from these embryos. It is likely that only small proportion of cells in these embryos are infected at the time of explantation. If we look upon the infected embryo as a clone arising from a single infected cell it is evident that uninfected daughter cells may segregate from this cell under some conditions. Under these conditions a close integration between viral and cell genomes is excluded.

Presence of RIF in unfertilized eggs: Viremic hens were separated from roosters for over a month and their infertile eggs collected within a few hours of laying.

Yolk was tested for the presence of RIF by adding 0.1 ml to RSV-sensitive cultures, and challenging the cultures at two sucessive transfers.

TABLE 6

RIF IN UNFERTILIZED EGGS

	Relative sensitivity to RSV after 1st transfer	Relative sensitivity to RSV after 2nd transfer
Egg No. 1 yolk	0.47	0.005
Egg No. 2 yolk	0.15	0.005
Egg No. 3 yolk	0.6	0.005
Egg No. 4 yolk	1.0	0.01
Control	1.0	1.0

Yolk was obtained from unfertilized eggs of viremic females and tested for RIF by the standard procedure.

The result in Table 6 shows that RIF was present in the yolk of unfertilized eggs. The fact that a fairly substantial level of resistance was attained at the end of a single transfer, indicates that there may be as many as 10^6 infectious units of RIF per ml of yolk. The average volume of yolk is about 17 ml, indicating the presence of more than 10^7 infectious units of RIF in the unfertilized egg. This estimate is a minimum, as the egg requires about 24 hours to traverse the oviduct and uterus. During this time considerable degeneration of the germinal vesicle and thermal inactivation of virus could occur.

Discussion.—The parental chickens in this study can be divided into two classes, the viremics and the non-viremics. Viremic birds remained viremic for the entire period of ten months. There was no sign of a decrease in virus titer at any time during this period. Nor was there any indication of antibody formation to RIF. Histological examination of these birds showed no evidence of excessive stimulation of the lymphoid system. It seems likely, therefore, that the viremic birds were immunologically tolerant to RIF. Since it is shown that RIF is congenitally transmitted, it is likely that the viremic birds became tolerant by virtue of heavy intraembryonic infection.

Neutralizing antibodies to RIF could be detected in the sera of all the nonviremic adults. The antibody titers in the adult sera remained relatively constant throughout the experimental period. The constancy of antibody level over a long period of time suggests that RIF continues to multiply in the immune animal and thus provides a continuous stimulus for antibody production. Support for this suggestion comes from the finding that hens with no detectable viremia and a persistently high titer of serum antibody, such as hens No. 19 and No. 60, can produce heavily infected embryos. Thus the multiplication of RIF in the ova can be detected through cultivation *in vitro* of cells from the infected embryo. If the ova had been homogenized and assayed for virus it is likely that any mature virus present would have been inactivated by antibody from the yolk and blood. Since virus can be detected in the ova of some immune birds by cultivation of cells from the embryo, it is likely that it can be found by direct cultivation of various organs of many birds with antibody.

A clear pattern for congenital transmission of RIF emerges from the present study. All viremic hens and some nonviremic hens transmit the infection through the ovum to a high proportion of their offspring. Viremic roosters, on the other hand, are unable to transmit the infection to the egg in spite of the fact that many of the cells of the testes are infected. This failure may simply be a reflection of the

difficulty of infecting the mature ovum, since recent attempts by us to deliberately infect the freshly laid fertile egg through the yolk have consistently failed. It might, however, be due to the loss of virus from spermatozoa as a result of the shedding of cytoplasm and the marked loss of RNA which accompanies spermatogenesis.[6]

All the available experimental evidence shows that both viral antigen and mature virus particles of the RNA-containing chicken tumor viruses are restricted to the cytoplasm.[7] The failure of male transmission is the first suggestion that the viral genome may also be restricted to the cytoplasm. This evidence is complemented by the presence of high concentrations of virus in the unfertilized female gamete, a single cell with large amounts of cytoplasm, and the uniform success of congenital transmission by viremic females. It cannot be considered as proved that the viral genome is in the cytoplasm, since RNA is lost from the nucleus as well as from the cytoplasm during spermatogenesis.[6] However, the experiments tend to rule out the type of intimate association between the viral genome and host-cell chromosomes which occurs in lysogenic bacteria.

The finding that embryos may develop in a perfectly normal fashion despite infection of a high proportion of their cells is a striking demonstration of the avirulence of RIF at the cellular level. The visceral tumors which characterize lymphomatosis are not produced even among congenitally infected birds until reproductive age, and then in only a fraction of the infected birds. This relatively benign behavior would, of course, favor the perpetuation of RIF in nature since the congenitally infected birds, being viremic, would be expected to transmit the infection to their progeny. Furthermore, they serve as a continuous and rich source of virus for spread of the infection to other birds by contact. Thus the cellular avirulence of RIF permits the forging of a chain of natural history which begins with congenital transmission of RIF, followed by immunological tolerance, which in turn permits further congenital transmission as well as wide dissemination of the agent.

Summary.—Sera were collected by repeated bleeding over a period of ten months from a flock of chickens which had been selectively bred for a high incidence of lymphomatosis. The sera were assayed for the presence of RIF, a virus indistinguishable from lymphomatosis virus. The sera were also tested for antibodies to RIF and to RSV which is immunologically related to RIF. Seven of the 26 birds had high concentrations of RIF and these showed no appreciable change in virus titer throughout the period of study. None of the viremic birds had antibody to RIF and only one had antibody to RSV. The remaining 19 birds were nonviremic but had antibody of RIF, and 15 had antibody to RSV. The antibody titer remained relatively constant during the period of investigation.

Three of the four viremic hens were fertile and most of their embryos proved to be infected. There was no indication that the three viremic roosters in the study transmitted virus to their progeny despite the fact that a high proportion of cells in the testes were actively producing virus. Only one of the 12 fertile nonviremic hens regularly infected her progeny. This single case, however, demonstrated that RIF could continue to multiply in the ova despite the presence of high concentrations of antibody.

High proportions of cells in most congenitally infected embryos were found to be actively producing virus. It was held likely that the persistent high viremia in adults was the result of the early exposure of birds to high concentrations of virus

leading to an immunological tolerance of the virus.

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*IMMEDIATE OOPHORECTOMY AND ADRENALECTOMY IN METASTATIC BREAST CANCER**

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Very little improvment in the treatment of cancer of the breast has appeared since the work of Halsted[1] and Meyer[2] and colleagues over sixty years ago. When at operation the axillary glands are found to be involved, the five-year survival rate is tragically low, 20–30 per cent. Attempts are being made to improve these results by more radical removal of lymph glands and channels above the clavicle and within the chest.[3, 4] In disillusionment some have abandoned radical mastectomy in favor of simple mastectomy and irradiation therapy.[5] The value of these procedures has not yet been determined.

A new approach to this old problem was made by Huggins and Bergenstal[6] in 1951. Influenced no doubt by the studies of C. R. Moore who demonstrated that the growth of the prostate gland is dependent upon hormones from the testes, Huggins removed the testes from patients with cancer of the prostate and widespread metastases. He theorized that such cancers might also be hormone dependent. The prompt relief of symptoms and prolongation of life secured by many patients confirmed this view.

It was a natural extension of these ideas that prompted Huggins to remove the ovaries and adrenal glands in patients with extensive metastases from breast cancer. Beatson[7] in 1896 had reported that regression of mammary carcinoma might follow removal of the ovaries, but little attention was paid to this significant observation. Huggins appreciated that steroids from the adrenal cortex could also promote the growth of secondary sex organs and concluded that both ovaries and adrenals ought to be extirpated in order to remove more effectively hormone stimulation of cancers of breast origin. With the isolation of cortisone and the development of an adequate substitution therapy for adrenal deficiency, this method of treatment became practical. Both Dao and Huggins[8] and Cade[9] have reported

marked improvement in 40 per cent of patients with advanced cancer of the breast treated by bilateral oophorectomy and adrenalectomy. For the most part, patients treated by this method had already developed massive metastatic involvement and the procedure was carried out as a last resort after palliation had been attempted in other ways.

In general, it has been found that patients in whom histological examination has revealed a well differentiated tumor secure a more favorable response than patients with more anaplastic types of cells. Exceptions, however, occur and in many patients different areas of the tumor display widely divergent histological pictures. In one area well differentiated glandular tissue may be reproduced while in others no such structure can be discerned.

It has been commonly assumed that carcinomas of the breast that are hormone dependent are so in the beginning and this property persists in the original tumor and its metastases throughout the course of the disease. Conversely, other tumors are entirely autonomous from the beginning and are not dependent upon the hormones of the ovaries and adrenal glands for growth or development at any stage. While this, in fact, may be the case, there is some evidence which suggests that most, if not all, cancers of the breast are hormone dependent at the beginning and later on lose this property as a result of continuing mutation under the influence of cancer inciting agents.

The condition of patients with carcinoma of the breast and metastases, who have experienced palliation after the removal of the ovaries and adrenal glands, deteriorates quite rapidly when growth in the dormant metastases is resumed. In some cases this is doubtless due to incomplete removal of the adrenal glands or ovaries or to functional development of aberrant tissue. An alternative hypothesis[10] postulates that this rapid growth is due to the appearance of mutant cancer cells that can grow in the absence of the hormones from the ovaries and adrenal glands. As an extension of this hypothesis, it is conceivable that the carcinogenic stimulus produces first a modification in the genetic composition of the cell sufficient to make it malignant but not yet sufficient to destroy its dependence upon these hormones for growth. Under the continuing influence of the carcinogenic agent, the malignant cell becomes autonomous and loses its hormone dependency.

The situation may be similar to the appearance of antibiotic resistant microorganisms in cultures which are held in check for varying periods by streptomycin or other antibiotics. These resistant organisms appear to be mutants that can survive in the presence of the antibiotic or may even require it for satisfactory growth. Perhaps the period of palliation in patients with metastatic cancer of the breast terminates with the appearance of mutant cancer cells that can grow in the absence of the hormones from the ovaries and adrenal glands. If this is true, the opportunity for the development of such mutants should be much greater when a large mass of carcinoma cells is actively dividing than when metastatic islands are of microscopic size. Removal of the ovaries and adrenals at the earliest possible moment should, on this basis, prolong the period of palliation and might even prevent the appearance of such mutant cancer cells.

These ideas appear to be supported by experience in the clinic.[11] Twenty-four consecutive patients with recurrent or metastatic cancer of the breast were treated by bilateral oophorectomy and adrenalectomy when first seen. In approximately

50 per cent of the patients, the distant metastases were small, and had not yet caused impairment of health, while the remainder were bedridden with extensive carcinomatosis. Fourteen, or 58 per cent, secured marked subjective and objective improvement and survived for an average of 50 months. Six patients are still living, an average of 74 months since the adrenalectomy. Two patients with disabling metastatic breast carcinoma are living and in good health nine and ten years, respectively, since the adrenalectomy and oophorectomy. These results are better than those reported by most writers in that a larger proportion of the patients have secured a favorable response and the period of palliation has been definitely longer.

In one patient[10] not included in this series, the ovaries and adrenal glands were removed shortly after radical mastectomy had been performed for carcinoma of the breast. The specimen removed revealed a large highly invasive tumor with cancerous involvement of three fourths of the axillary lymph glands, and permeation of cancer cells into the lymphatics, blood vessels, and subcutaneous tissue. Distant metastases were not demonstrated but were believed to be present. Previous experience with breast carcinomas of this type has demonstrated that 90–95 per cent of them fail to survive 3 years. However, this patient has remained well without evidence of recurrence and has continued her usual activities for over eight years.

While clearly the number of breast cancer patients in this category, treated at an early stage by removal of the ovaries and adrenal glands, is too small for far-reaching conclusions, the findings so far are encouraging and suggest that this procedure be employed as soon as metastatic lesions from breast carcinoma are diagnosed or are believed to be present.

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ON A NEW COMPUTATIONAL SOLUTION OF TIME-DEPENDENT TRANSPORT PROCESSES, I. ONE-DIMENSIONAL CASE

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1. *Introduction.*—Stimulated by the fundamental work of Ambarzumian and Chandrasekhar in the field of radiative transfer (see ref. 1), there has been a good deal of effort in recent years devoted to the transformation of linear functional equations subject to boundary conditions into nonlinear functional equations subject only to initial conditions in space and time coordinates. For techniques based directly upon the physical processes, see Chandrasekhar,[1] Bellman and Kalaba,[2] Bellman, Kalaba, and Wing,[3] Bellman and Harris,[4] and the book by Harris;[5] for purely analytic approaches, see Wing,[6] Bellman and Kalaba,[7] and Mullikin.[8]

The approach of invariant imbedding of Bellman and Kalaba, which yields at one stroke the reflected and transmitted fluxes as functions of basic physical dimensions such as thickness, has been carried out for the steady-state case and will soon appear.[7, 9] As a first step toward the computational solution of the corresponding problem for the time-dependent case, we decided to study the reflected flux from a one-dimensional rod for the case of neutron transport.

In the course of obtaining a numerical solution, we encounter the problem of inverting the Laplace transform numerically. The method we employ is quite simple and has apparently worked quite well. We feel that it will be equally applicable to the more general problems which we shall discuss in subsequent communications.

2. *The Nonlinear Equation.*—Using the physical model discussed in reference 10, the techniques of invariant imbedding yield the normalized equation for reflected flux from a rod of length x over a time t,

$$\frac{\partial U}{\partial x} + 2\frac{\partial U}{\partial t} = 1 + \int_0^t u(x,z)U(x, t - z)dz, \qquad U(x,0) = 0, \quad U(0,t) = 0. \tag{2.1}$$

Here $u = \partial U/\partial t$, and we have the initial conditions $U(x,0) = U(0,t) = 0$. A discussion of the existence and uniqueness of solutions of this equation is contained in reference 11. An explicit analytic representation of the solution in terms of a contour integral is easily obtained, since the Laplace transform,

$$F(x,s) = \int_0^\infty e^{-st}u(x,t)dt, \tag{2.2}$$

satisfies the Riccati equation,

$$\frac{dF}{dx} = F^2 - 2sF + 1, \qquad F(0,s) = 0. \tag{2.3}$$

The explicit form of F is readily found. Using the standard inversion formula and standard contour shifting, it is easy to obtain the asymptotic behavior of the function $U(x,t)$ as $t \to \infty$. For $x < \pi/2$, the critical length, we have $U(x,t) \sim \tan x$, for $x = \pi/2$, $U(x,t) \sim t$, and for $x > \pi/2$, asymptotically in t, $U(x,t)$ grows exponentially at a rate dependent upon x.[11]

It is important to begin computational studies with a problem for which some information concerning the solution is available in order to check the accuracy of the numerical techniques. As will be seen from the graphical results below, we obtain excellent agreement with the analytic results as far as the calculations have been carried.

A direct attack upon (2.1) does not seem profitable because of the appearance of the two partial derivatives and of the convolution integral. Instead, we shall base our computational solution upon (2.3) and a numerical inversion of the Laplace transform.

3. *Numerical Inversion of the Laplace Transform.*—The relation of (2.2) may be written

$$\int_0 e^{-st}U(x,t)dt = \frac{F(x,s)}{s}, \quad (3.1)$$

upon integrating by parts. Making the change of variable $e^{-t} = r$, this formula becomes

$$\int_0^1 r^{s-1}U(x, \log 1/r)dr = \frac{F(x,s)}{s}. \quad (3.2)$$

Applying Gaussian quadrature with N abscissae (suitably adjusted for $0 \leq r \leq 1$), we obtain the set of approximate equations,

$$\sum_{i=1}^{N} w_i r_i^{s-1} g(r_i) = \frac{F(x,s)}{s}, \quad (3.3)$$

where $g(r) = U(x, \log 1/r)$ for fixed x. Allowing s to assume the N values $s = 1,2,\ldots,N$, we obtain N simultaneous equations for the N unknown values $g(r_i)$.

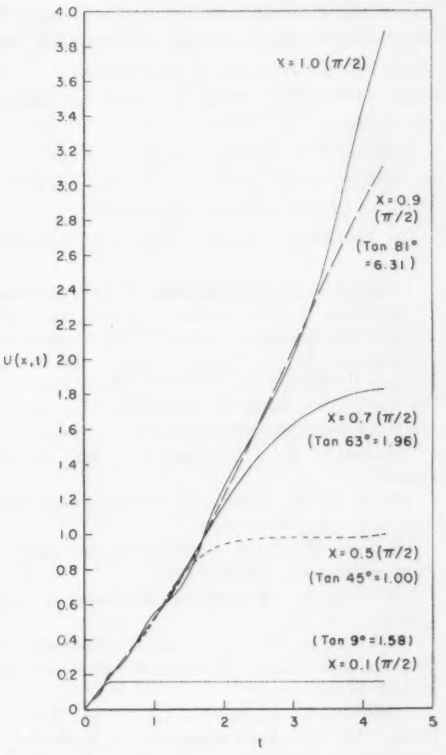

FIG. 1.—Reflected fluxes as functions of time for rods of various lengths.

Testing this technique for simple functions such as $g = 1$ and $g = r^{1/2}$, we found excellent agreement with maximum error of the order of 1 in 200 for $N = 10$. Considering the fact that the function we are examining, $U(x,t)$, is monotone increasing in both x and t, we feel that this accuracy is sufficient for our investigations. After all, the physical models we study do not possess these accuracies, and in many cases we are more interested in qualitative than quantitative phenomena.

An interesting feature of the method is that we obtain most accurate values of $U(x,t)$ for small values of t. In many investigations, this is the region of greatest interest.

There appeared to be no advantage in allowing s to assume more than N values and then determining $g(r_i)$ via least squares.

4. *Computational Aspects.*—Letting s assume the values $1,2,\ldots,N$, we obtain, using an obvious notation, the system of simultaneous differential equations,

$$\frac{dF_k}{dx} = F_k^2 - 2kF_k + 1, \qquad F_k(0) = 0, \quad k = 1,2,\ldots,N. \tag{4.1}$$

As soon as the values of the F_k for one value of x are computed, we employ the preceding inversion procedure to obtain the values of $U(x, \log 1/r_i)$. This procedure is repeated step by step with the result that there is a very small demand upon the fast memory of the computer. This is not a particularly important point here, but would be in the more interesting cases involving energy and angular dependence.

Taking $N = 10$, the time required to print out the results at intervals of 0.05π in x, with a computation of intervals of 0.005π in x, is about one minute, using a Fortran program for an IBM-7090.

5. *Graphical Results.*—The results obtained are given in the graphs of Figure 1.

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QUANTUM VARIABLES AND THE ACTION PRINCIPLE

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In previous communications, a classification of quantum degrees of freedom by a prime integer ν has been given,[1] and a quantum action principle has been constructed[2] for $\nu = \infty$. Can a quantum action principle be devised for other types of quantum variables? We shall examine this question for the simplest quantum degree of freedom, $\nu = 2$.

Let us consider first a single degree of freedom of this type. The operator basis is generated by the complementary pair of Hermitian operators

$$\xi_1 = 2^{-1/2}\sigma_1, \quad \xi_2 = 2^{-1/2}\sigma_2$$

which obey

$$\xi_k\xi_l + \xi_l\xi_k = \{\xi_k, \xi_l\} = \delta_{kl}.$$

The basis is completed by the unit operator and the product

$$\xi_3 = 2^{-1/2}\sigma_3 = -i2^{1/2}\xi_1\xi_2.$$

We have remarked[1] upon the well-known connection between the σ_k, $k = 1, 2, 3$, and three-dimensional rotations. In particular, the most general unitary operator that differs infinitesimally from unity is, apart from a phase factor, of the form

$$U(\delta\omega) = 1 + {}^1/_2 i\delta\omega\cdot\sigma,$$

and the corresponding operator transformation

$$\delta\sigma = U^{-1}\sigma U = \sigma - \delta\sigma$$

is the three-dimensional infinitesimal rotation

$$\delta\sigma = \delta\omega \times \sigma.$$

Accordingly, an infinitesimal transformation that varies ξ_1 and not ξ_2 can only be a rotation about the second axis,

$$\delta\xi_1 = \delta\omega_2\xi_3, \quad \delta\xi_2 = 0.$$

The corresponding infinitesimal generator is

$$G_1 = {}^1/_2\delta\omega_2\sigma_2 = i\xi_1\delta\xi_1 = -i\delta\xi_1\xi_1.$$

Similarly,

$$\delta\xi_1 = 0, \quad \delta\xi_2 = -\delta\omega_1\xi_3$$

is generated by

$$G_2 = i\xi_2\delta\xi_2 = -i\delta\xi_2\xi_2,$$

and the generator for the combination of these elementary transformations is ($k = 1, 2$)

$$G = i\sum_k \xi_k \delta\xi_k = -i\sum_k \delta\xi_k \xi_k.$$

It is evident that G_1 and G_2 must be supplemented by

$$G_3 = {}^1/_2 \delta\omega_3 \sigma_3$$

to form the infinitesimal generators of a unitary group, which is isomorphic to the three-dimensional rotation group. The transformation induced by G_3 is

$$\delta\xi_1 = -\delta\omega_3 \xi_2, \quad \delta\xi_2 = \delta\omega_3 \xi_1,$$

and

$$G_3 = {}^1/_2 i(\xi_1 \delta\xi_1 + \xi_2 \delta\xi_2).$$

Thus, the concentration on the complementary pair of operators ξ_1 and ξ_2 does not give a symmetrical expression to the underlying three-dimensional rotation group. This is rectified somewhat by using, for those special transformations in which ξ_1 and ξ_2 are changed independently, the generator of the variations ${}^1/_2\delta\xi_k$, $k = 1, 2$,

$$G_\xi = {}^1/_2 i\sum_k \xi_k \delta\xi_k$$

An arbitrary infinitesimal unitary transformation is described by the transformation function

$$\langle \tau + d\tau | \tau \rangle = \langle | [1 + i d\tau G(\xi, \tau)] | \rangle.$$

Infinitesimal variations in τ, $\tau + d\tau$, and the structure of G induce

$$\delta''\langle \tau + d\tau | \tau \rangle = i\langle | \delta''[d\tau G(\xi, \tau)] | \rangle = i\langle \tau + d\tau | \delta''[d\tau G(\xi(\tau), \tau)] | \tau \rangle.$$

To this we add δ', the transformations generated by G_ξ, performed independently but continuously in τ, on the states $\langle \tau + d\tau |$ and $| \tau \rangle$,

$$\delta'\langle \tau + d\tau | \tau \rangle = i\langle \tau + d\tau | [G_\xi(\tau + d\tau) - G_\xi(\tau)] | \tau \rangle.$$

Here, $G_\xi(\tau + d\tau) - G_\xi(\tau) = -{}^1/_2 i\sum \delta\xi_k(\tau + d\tau)\xi_k(\tau + d\tau) - {}^1/_2 i\sum \xi_k(\tau)\delta\xi_k(\tau)$

$$= -{}^1/_2 i\sum [\delta\xi_k(\tau + d\tau)\xi_k(\tau) + \xi_k(\tau + d\tau)\delta\xi_k(\tau)] + {}^1/_2\sum [\delta\xi_k, [\xi_k, d\tau G]],$$

or

$$G_\xi(\tau + d\tau) - G_\xi(\tau) = \delta'[-{}^1/_2 i\sum \xi_k(\tau + d\tau)\xi_k(\tau) + d\tau G(\xi(\tau), \tau)] - {}^1/_2\sum [\xi_k, [d\tau G, \delta\xi_k]],$$

where δ', in its effect upon operators, refers to the special variations $\delta\xi_k$, $k = 1, 2$, performed independently but continuously at τ and $\tau + d\tau$.

It is only if the last term is zero that one obtains the quantum action principle $[\delta = \delta' + \delta'']$

$$\delta\langle \tau + d\tau | \tau \rangle = i\langle \tau + d\tau | \delta[W] | \tau \rangle,$$

with

$$W(\tau + d\tau, \tau) = -{}^1/_2 i\sum_k \xi_k(\tau + d\tau)\xi_k(\tau) + d\tau G(\xi(\tau), \tau).$$

Since the special variation is such that $\delta\xi_1$ and $\delta\xi_2$ are arbitrary multiples of ξ_3, it

is necessary that $[G, \xi_3]$ commute with ξ_1 and ξ_2. Hence this commutator must be a multiple of the unit operator, which multiple can only be zero, since the trace of the commutator vanishes or, alternately, as required by $[G, \xi_3^2] = 0$. For an action principle formulation to be feasible, it is thus necessary and sufficient that

$$[G, \xi_1\xi_2] = 0.$$

Terms in ξ_1 and ξ_2 are thereby excluded from G, which restriction is also conveyed by the statement that a permissible G must be an even function of the ξ_k, $k = 1, 2$. Apart from multiples of the unit operator, generating phase transformations, the only allowed generator is $\xi_1\xi_2$, which geometrically is a rotation about the third axis.

It should be noted that the class of variations δ' can be extended to include the one generated by G_3, without reference to the structure of G. Thus,

$$G_3(\tau + d\tau) - G_3(\tau) = -{}^1/_2 i\sum[\delta\xi_k(\tau + d\tau)\xi_k(\tau) + \xi_k(\tau + d\tau)\delta\xi_k(\tau)] + {}^1/_2\sum[\delta\xi_k, [\xi_k, d\tau G]],$$

where the latter term equals

$${}^1/_2\delta\omega_3[\xi_1, [\xi_2, d\tau G]] - {}^1/_2\delta\omega_3[\xi_2, [\xi_1, d\tau G]] = -{}^1/_2\delta\omega_3[d\tau G, [\xi_1, \xi_2]] = 1/i[d\tau G, G_3],$$

and therefore, for this kind of δ' variation,

$$G_3(\tau + d\tau) - G_3(\tau) = \delta'[-{}^1/_2 i\sum\xi_k(\tau + d\tau)\xi_k(\tau) + d\tau G].$$

The action operator for a finite unitary transformation is

$$W_{12} = \int_{\tau_2}^{\tau_1} d\tau \left[-{}^1/_2 i\sum \frac{d\xi_k}{d\tau}\,\xi_k + G \right]$$

or, more symmetrically,

$$W_{12} = \int_{\tau_2}^{\tau_1} d\tau \left[{}^1/_4 i \sum_k \left(\xi_k \frac{d\xi_k}{d\tau} - \frac{d\xi_k}{d\tau}\,\xi_k \right) + G \right],$$

since W_{12} is only defined to within an additive constant, and

$$\frac{d}{d\tau}(\xi_k(\tau))^2 = 0.$$

The principle of stationary action

$$\delta[W_{12}] = G_1 - G_2,$$

which refers to a fixed form of the operator $G(\xi(\tau), \tau)$, expresses the requirement that a finite transformation emerge from the succession of infinitesimal transformations. It will be instructive to see how the properties of the quantum variables are conversely implied by this principle. The discussion will be given without explicit reference to the single pair of variables associated with one degree of freedom, since it is of greater generality.

The bilinear concomitant

$$\delta({}^1/_4 i\sum[\xi_k, d\xi_k] + Gd\tau) - d({}^1/_4 i\sum[\xi_k, \delta\xi_k] + G\delta\tau) = {}^1/_2 i\sum[\delta\xi_k, d\xi_k] + \delta G d\tau - dG\delta\tau$$

shows that

$$\delta G = \frac{dG}{d\tau}\,\delta\tau - {}^1/_2 i \sum \left[\delta\xi_k, \frac{d\xi_k}{d\tau}\right]$$

and gives

$$G_{1,2} = {}^1/_4 i \sum [\xi_k, \delta\xi_k] + G\delta\tau\big|_{\tau_1, \tau_2}.$$

The generator term $G\delta\tau$ evidently restates the transformation significance of the operator G. The effect upon operators is conveyed by the infinitesimal unitary transformation

$$\bar{F}(\xi(\tau), \tau) = (1 - iG\delta\tau)F(\xi(\tau), \tau)(1 + iG\delta\tau) = F(\xi(\tau + \delta\tau), \tau),$$

which is the general equation of motion,

$$\frac{dF}{d\tau} = \frac{\partial F}{\partial \tau} - \frac{1}{i}[F, G].$$

Let us take $\delta\xi_k$ to be a special variation, which we characterize by the following properties: (1) Each $\delta\xi_k$ anticommutes with every ξ_l,

$$\{\delta\xi_k, \xi_l\} = 0;$$

(2) the $\delta\xi_k(\tau)$ have no implicit τ dependence; and (3) every $\delta\xi_k$ is an arbitrary infinitesimal numerical multiple of a common nonsingular operator, which does not vary with τ. The second basic property asserts that

$$[\delta\xi_k, G] = 0$$

which restricts G to be an even function of the ξ_k, in virtue of the anticommutativity of the special variations with each member of this set. Furthermore, the τ derivatives of the special variations are also anticommutative with the ξ_k, according to property (3), and therefore,

$$\sum {}^1/_2 \left[\delta\xi_k, \frac{d\xi_k}{d\tau}\right] = \frac{d}{d\tau}\sum {}^1/_2[\delta\xi_k, \xi_k] - \sum {}^1/_2 \left[\frac{\partial\delta\xi_k}{\partial\tau}, \xi_k\right] =$$

$$\frac{d}{d\tau}\sum \delta\xi_k\xi_k - \sum \frac{\partial\delta\xi_k}{\partial\tau}\,\xi_k = \sum \delta\xi_k \frac{d\xi_k}{d\tau} = -\sum \frac{d\xi_k}{d\tau}\,\delta\xi_k.$$

Then, if we write

$$\delta G - \frac{\partial G}{\partial\tau}\,\delta\tau = \sum \delta\xi_k \frac{\partial_l G}{\partial\xi_k} = \sum \frac{\partial_r G}{\partial\xi_k}\,\delta\xi_k,$$

which defines the left and right derivatives of G with respect to the ξ_k, we get

$$\frac{dG}{d\tau} = \frac{\partial G}{\partial\tau},$$

which is consistent with the general equation of motion, and

$$\sum \delta\xi_k \left(i\frac{d\xi_k}{d\tau} + \frac{\partial_l G}{\partial\xi_k}\right) = \sum \left(-i\frac{d\xi_k}{d\tau} + \frac{\partial_r G}{\partial\xi_k}\right)\delta\xi_k = 0.$$

The nonsingular operator contained in every special variation can be cancelled from the latter equation, and the arbitrary numerical factor in each $\delta\xi_k(\tau)$ implies that

$$i\frac{d\xi_k}{d\tau} = \frac{\partial_r G}{\partial \xi_k} = -\frac{\partial_l G}{\partial \xi_k}.$$

In the opposite signs of left and right derivatives, we recognize the even property of the function G.

On comparing the two forms of the equation of motion for ξ_k we see that

$$[G, \xi_k] = \frac{\partial_r G}{\partial \xi_k}.$$

If we reintroduce the special variations, this reads

$$\frac{1}{i}[G, {}^1/_2 i \sum \xi_k \delta\xi_k] = \sum \frac{\partial_r G}{\partial \xi_k} {}^1/_2\delta\xi_k.$$

The left side is just the change induced in G by G_ξ, the generator of the special variations, which also appears in $G_{1,2}$, while the right side gives the result in terms of changes of the ξ_k by ${}^1/_2\delta\xi_k$. Since G is an arbitrary even function of the ξ_k, we accept this as the general interpretation of the transformation generated by G_ξ, which then asserts of any operator F that

$$\frac{1}{i}[F, G_\xi] = \sum \frac{\partial_r F}{\partial \xi_k} {}^1/_2\delta\xi_k = \sum {}^1/_2\delta\xi_k \frac{\partial_l F}{\partial \xi_k}.$$

The implication for an odd function of the ξ_k is

$$\{F, \xi_k\} = \frac{\partial_r F}{\partial \xi_k} = \frac{\partial_l F}{\partial \xi_k},$$

and the particular choice $F = \xi_l$ gives the basic operator properties of these quantum variables

$$\{\xi_k, \xi_l\} = \delta_{kl}.$$

In this way, we verify that the quantum action principle gives a consistent account of all the characteristics of the given type of quantum variable.

The operator basis of a single degree of freedom is used in a different manner when the object of study is the three-dimensional rotation group rather than transformations of the pair of complementary physical properties. The generator of the infinitesimal rotation $\delta\sigma = \delta\omega \times \sigma$ is

$$G_\omega = {}^1/_2\delta\omega\cdot\sigma = {}^1/_4 i\sigma\cdot\delta\sigma = -{}^1/_4 i\delta\sigma\cdot\sigma.$$

On applying this transformation independently but continuously to the states of the transformation function $\langle\tau + d\tau|\,\tau\rangle$, we encounter

$$G_\omega(\tau + d\tau) - G_\omega(\tau) = -{}^1/_4 i\delta\sigma(\tau + d\tau)\cdot\sigma(\tau) - {}^1/_4 i\sigma(\tau + d\tau)\cdot\delta\sigma(\tau) + {}^1/_4[\delta\sigma\cdot, [\sigma, d\tau G]].$$

The identity,

$$[\delta\sigma\cdot, [\sigma, d\tau G]] - [\sigma\cdot, [\delta\sigma, d\tau G]] = [d\tau G, [\sigma\cdot, \delta\sigma]],$$

combined with the equivalence of the two left-hand terms, both of which equal

$$\delta\omega\cdot\sigma\times,\ [\sigma,\ d\tau G],$$

then gives

$$G_\omega(\tau + d\tau) - G_\omega(\tau) = \delta'[-{}^1/_4 i\sigma(\tau + d\tau)\cdot\sigma(\tau) + d\tau G(\sigma(\tau),\ \tau)],$$

where δ' describes the independent operator variations $\delta\sigma(\tau) = \delta\omega(\tau) \times \sigma(\tau)$ at τ and $\tau + d\tau$. If we add the effect of independent variations of τ, $\tau + d\tau$, and the structure of G [δ''], we obtain an action principle[3] without restrictions on the form of G.

The action operator for a finite transformation is

$$W_{12} = \int_{\tau_2}^{\tau_1} d\tau \left[-{}^1/_4 i \frac{d\sigma}{d\tau}\cdot\sigma + G \right]$$

$$= \int_{\tau_2}^{\tau_1} d\tau \left[{}^1/_8 i \left(\sigma\cdot\frac{d\sigma}{d\tau} - \frac{d\sigma}{d\tau}\cdot\sigma \right) + G \right].$$

The most general form for G, describing phase transformations and rotations, is

$$G = g(\tau) + {}^1/_2\sigma\cdot\frac{d\omega(\tau)}{d\tau}.$$

We shall be content to verify that the principle of stationary action reproduces the equations of motion that also follow directly from the significance of G,

$$\frac{d\sigma}{d\tau} = = -\frac{1}{i}\,[\sigma,\ G] = \sigma \times \frac{d\omega}{d\tau}.$$

The action principle asserts that

$$\frac{1}{4}\,i\left[\delta\sigma\cdot,\ \frac{d\sigma}{d\tau}\right] + \delta G - \frac{\partial G}{\partial\tau}\,\delta\tau = 0,$$

where the operator variations are arbitrary infinitesimal rotations, $\delta\sigma = \delta\omega \times \sigma$. Hence the equations of motion are

$$-{}^1/_2 i\left(\sigma \times \frac{d\sigma}{d\tau} + \frac{d\sigma}{d\tau} \times \sigma\right) = \sigma \times \frac{d\omega}{d\tau},$$

which appear in the anticipated form on remarking that the left-hand side of the latter equation equals

$$\frac{d}{d\tau}\,\frac{1}{2i}\,\sigma \times \sigma = \frac{d}{d\tau}\,\sigma.$$

The extension to n degrees of freedom of type $\nu = 2$ requires some discussion. At first sight, the procedure would seem to be straightforward. Operators associated with different degrees of freedom are commutative, and the infinitesimal generators of independent transformations are additive, which implies an action operator of the previous form with the summation extended over the n pairs of complementary variables. But we should also conclude that G must be an even

function of the complementary variables associated with each degree of freedom separately, and this is an unnecessarily strong restriction.

To loosen the stringency of the condition for the validity of the action principle, we replace the relationship of commutativity between different degrees of freedom by one of anticommutativity. Let $\xi^{(\alpha)}{}_{1,2,3}$ be the operators associated with the α degree of freedom. We define

$$\xi_1 = \xi_1{}^{(1)}, \quad \xi_2 = \xi_2{}^{(1)}$$

$$\xi_{2k+1,\,2} = \prod_{\alpha=1}^{k} (2^{1/2}\xi_3{}^{(\alpha)})\xi_{1,\,2}{}^{(k+1)}, \qquad k = 1, 2, \ldots n-1$$

and this set of $2n$ Hermitian operators obeys

$$\{\xi_k, \xi_l\} = \delta_{kl}, \qquad k, l = 1 \ldots 2n.$$

The inverse construction is

$$\xi_{1,\,2}{}^{(k+1)} = (-2i)^k \xi_1 \xi_2 \ldots \xi_{2k}\xi_{2k+1,\,2},$$

and in

$$\xi_{2n+1} = (-2i)^n \xi_1 \xi_2 \ldots \xi_{2n} \cdot 2^{-1/2},$$

we have an operator that extends by one the set of anticommuting Hermitian operators with squares equal to $1/2$. In particular this operator anticommutes with every ξ_k, $k = 1 \ldots 2n$.

An infinitesimal transformation that alters only ξ_k must be such that

$$\{\delta\xi_k, \xi_l\} = 0 \qquad l = 1 \ldots 2n,$$

which identifies $\delta\xi_k$ as an infinitesimal numerical multiple of ξ_{2n+1}. We shall write

$$\begin{aligned} {}^1/_2\delta\xi_k &= -\delta\omega_k \xi_{2n+1} \\ &= \frac{1}{i}[\xi_k, G_\xi] \end{aligned}$$

and the generator of all these special variations is

$$G_\xi = \sum_{k=1}^{2n} \delta\omega_k \frac{1}{i} \xi_k \xi_{2n+1}.$$

The latter can also be written as

$$G_\xi = {}^1/_2 i \sum_{k=1}^{2n} \xi_k \delta\xi_k = -{}^1/_2 i \sum \delta\xi_k \xi_k.$$

On forming the commutator of two such generators, we get

$$\frac{1}{i}[G_\xi{}^{(1)}, G_\xi{}^{(2)}] = \sum_{k,\,l} {}^1/_2(\delta^{(1)}\omega_k \delta^{(2)}\omega_l - \delta^{(1)}\omega_l \delta^{(2)}\omega_k)\xi_{kl},$$

where the Hermitian operators

$$\xi_{kl} = \frac{1}{2i}[\xi_k, \xi_l] = -\xi_{lk}$$

are $n(2n-1)$ in number, and obey ($k \neq l$)

$$\xi_{kl}^2 = {}^1/_4.$$

The generators G_ξ and their commutators can thus be constructed from the basis provided by the $n(2n+1)$ operators ξ_{kl}, with k and l ranging from 1 to $2n+1$. And, since

$$1/i[\xi_{kl}, \xi_{pq}] = \delta_{lq}\xi_{kp} - \delta_{kq}\xi_{lp} + \delta_{kp}\xi_{lq} - \delta_{lp}\xi_{kq},$$

these operators are the generators of a unitary transformation group, which has the structure of the Euclidean rotation group in $2n+1$ dimensions. For $n > 1$, the operators ξ_{kl}, $k, l = 1 \ldots 2n+1$ can also be combined with the linearly independent set

$$\xi_{0k} = 2^{-1/2}\xi_k, \qquad k = 1 \ldots 2n+1$$

to form the $(n+1)(2n+1)$ generators of a similar group associated with rotations in $2n+2$ dimensions.

It should also be noted that

$$G_\omega = {}^1/_2 \sum_{k,\,l\,=\,1}^{2n} \delta\omega_{kl}\xi_{kl}, \qquad \delta\omega_{kl} = -\delta\omega_{lk}$$

induces the linear transformation

$$\delta\xi_k = \frac{1}{i}[\xi_k, G_\omega] = -\sum_{l=1}^{2n} \delta\omega_{kl}\xi_l,$$

and one can write

$$G_\omega = {}^1/_2 i \sum_{k=1}^{2n} \xi_k\delta\xi_k = -{}^1/_2 i \sum \delta\xi_k\xi_k.$$

These generators have the structure of the rotation group in $2n$ dimensions.

The discussion of the change induced in a transformation function $\langle \tau + d\tau | \tau \rangle$ by the special variations, applied independently but continuously at τ and $\tau + d\tau$, proceeds as in the special example $n = 1$, and leads to

$$\sum_{k=1}^{2n} [\xi_k, [d\tau G, \delta\xi_k]] = 0$$

for the condition that permits an action principle formulation. Each special variation is proportional to the single operator ξ_{2n+1}. It is necessary, therefore, that $[G, \xi_{2n+1}]$ commute with every ξ_k, $k = 1 \ldots 2n$, or equivalently, with each complementary pair of operators $\xi_{1,\,2}{}^{(\alpha)}$, $\alpha = 1 \ldots n$. Such an operator can only be a multiple of the unit operator and that multiple must be zero. Hence the infinitesimal generators of transformations that can be described by an action principle must commute with ξ_{2n+1}. Considered as a function of the anticommutative operator set ξ_k, $k = 1 \ldots 2n$ which generates the 2^{2n} dimensional operator basis, an admissible operator $G(\xi)$ must be even. This single condition replaces the set of n conditions that appear when commutativity is the relationship between different degrees of freedom. Of course, if the class of transformations under consideration is such that G is also an even function of some even-dimensional subsets of the ξ_k, one can

consistently adopt commutativity as the relationship between the various subsets. Incidentally, the commutativity of G with $\xi_{2n+1}(\tau)$ asserts that the latter operator does not vary with τ. Accordingly, the special variations $\delta\xi_k(\tau)$ have no implicit operator dependence upon τ and one concludes that the special variations are anticommutative with the $2n$ fundamental variables ξ_k, without reference to the associated τ values.

The action principle is also valid for the linear variations induced by G_ω, without regard to the structure of G. Thus,

$$G_\omega(\tau + d\tau) - G_\omega(\tau) = -{}^1/_2 i \sum [\delta\xi_k(\tau + d\tau)\xi_k(\tau) + \xi_k(\tau + d\tau)\delta\xi_k(\tau)] + {}^1/_2 \sum [\delta\xi_k, [\xi_k, d\tau G]],$$

and the last term equals

$$-{}^1/_4 \sum_{k,l} \delta\omega_{kl}[\xi_l, [\xi_k, d\tau G]] + {}^1/_4 \sum \delta\omega_{kl}[\xi_k, [\xi_l, d\tau G]] = \frac{1}{i}[d\tau G, G_\omega],$$

which gives

$$G_\omega(\tau + d\tau) - G_\omega(\tau) = \delta'[-{}^1/_2 i \sum_k \xi_k(\tau + d\tau)\xi_k(\tau) + d\tau G]$$

for this type of δ' variation.

The action operator associated with a finite transformation generalizes the form already encountered for $n = 1$,

$$W_{12} = \int_{\tau_2}^{\tau_1} d\tau \left[\frac{1}{4} i \sum_{k=1}^{2n} \left(\xi_k \frac{d\xi_k}{d\tau} - \frac{d\xi_k}{d\tau}\xi_k\right) + G(\xi(\tau), \tau)\right],$$

and the earlier discussion can be transferred intact. It may be useful, however, to emphasize that the special variations anticommute, not only with each $\xi_k(\tau)$, but also with $d\xi_k/d\tau$, since this property is independent of τ. Then it follows directly from the implication of the principle of stationary action,

$$\delta G = \frac{dG}{d\tau}\delta\tau - \frac{1}{2} i \sum_k \left[\delta\xi_k, \frac{d\xi_k}{d\tau}\right],$$

that the equations of motion are

$$i\frac{d\xi_k}{d\tau} = \frac{\partial_r G}{\partial\xi_k} = -\frac{\partial_l G}{\partial\xi_k}, \qquad k = 1 \ldots 2n.$$

The action principle is still severely restricted in a practical sense, for the generators of the special variations cannot be included in G since the operators $\xi_k\xi_{n+1}$ are odd functions of the $2n$ fundamental variables. It is for the purpose of circumventing this difficulty, and thereby of converting the action principle into an effective computation device, that we shall extend the number system by adjoining an exterior or Grassmann algebra.

[1] These PROCEEDINGS, **46,** 570 (1960).

[2] *Ibid.*, **46,** 883 (1960).

[3] The possibility of using the components of an angular momentum vector as variables in an action principle was pointed out to me by D. Volkov during the 1959 Conference on High Energy Physics held at Kiev, U.S.S.R.

*PHOTORECEPTORS IN THE AMPHIBIAN FRONTAL ORGAN**

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The frontal organ (stirnorgan or Stieda's organ) of frogs and toads has long been a physiological enigma, as has likewise its reptilian homologue, the parietal eye. Some investigators have suggested that the stirnorgan is sensory,[1] others that it is secretory;[2, 3] still others[4, 5] have found no evidence of either function. This study of the frontal organ in the tadpole of the Pacific Treefrog, *Hyla regilla*, indicates that it is photoreceptive.

Ultrastructure.—Electron microscopy of the stirnorgan of young larvae of *H. regilla* by Eakin and Westfall[6] reveals processes, extending into its tiny lumen, which are structurally similar to those of rods and cones in the retina of the lateral eye and to those recently discovered in the parietal eye of lizards.[7–10] Figure 1 shows a longitudinal section of one of these processes. The outer segment (*os*) contains an array of about 135 discs (*d*) or laminae formed apparently by infoldings of the surface membrane (see arrows). In this feature the outer segment resembles that of cones in the lateral eye of the anuran *Xenopus laevis*.[11] Many of the processes, such as the one figured, are short, stout, and bent in contradistinction to the long, straight filamentous outer segments of rods and cones of the vertebrate lateral eye and of receptors in the parietal eye. The inner segment (*is*), of which the distal part only is shown in Figure 1, contains many large elongate mitochondria (*m*) densely packed near the distal end of the segment as they are in the comparable region, the ellipsoid, of known photoreceptors; lipid droplets which are sometimes very large; osmiophilic granules (*g*, Fig. 2); and vesicles (*v*) of diverse sizes, some (see Fig. 2) reaching a diameter of 1μ and containing material (secretion?) slightly more electron dense than the surrounding cytoplasm. The connecting piece, a short, narrow part of the process joining inner and outer segments, is not shown in Figure 1 because the plane of section is to one side of it. Figure 2, however, illustrates the connecting piece (*cp*) of another process. A fibrillar apparatus, presumably derived from that of a cilium of an ependymal cell, arises from a basal granule or axial centriole (c_1) situated at the tip of the inner segment, passes through the connecting piece, and fans out into the clear matrix (*s*) of the outer segment. The apparatus is composed of nine peripheral fibrils (count based upon other micrographs), parts of which may be observed (*f*, Fig. 2). Adjacent to the axial centriole lies the oblique centriole, a short cylinder which appears in cross section (c_2, Fig. 2) to consist of nine peripheral fibers.

Stirnorganectomy.—Although the fine structure of the processes of the frontal organ just described is remarkably similar to that of rods and cones, evidence of light sensitivity is needed before the cells which bear them are designated photoreceptors. Bagnara[12] recently observed that the destruction of the epiphysis in tadpoles of *Xenopus laevis* eliminates the blanching reaction of the skin (concentration of pigment in the dermal melanophores of the body) commonly exhibited by amphibian larvae when placed in the dark. The frontal organ is known to be connected to the epiphysis by a nerve or pineal tract.[1–4, 13] I have traced it in larvae of *H. regilla*

from the posterior end of the frontal organ to the anterior tip of the epiphysis. Could the blanching response mediated by the epiphysis be triggered by stimuli from photoreceptors in the stirnorgan? To answer this question I removed the frontal organ of 40 larvae in Stage I[14] by means of microscissors,[15] and performed a sham operation to the side of the organ in 40 sibling tadpoles. As there is some

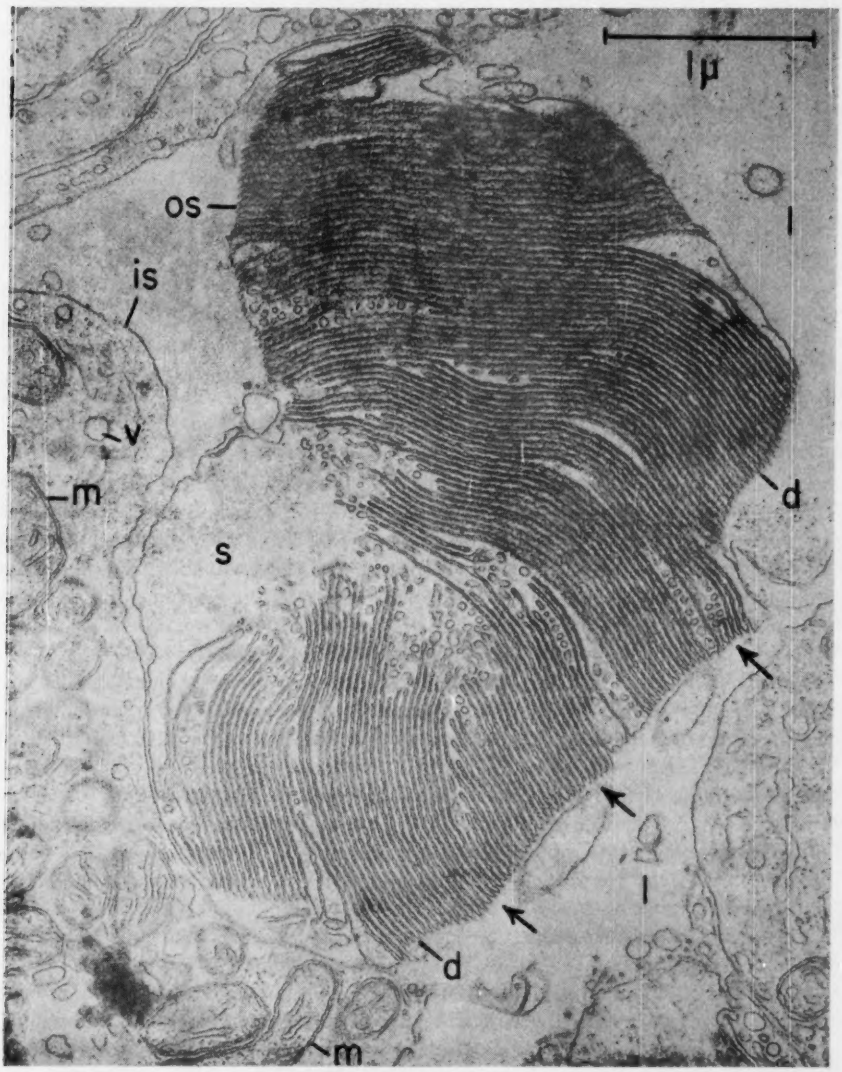

FIG. 1.—Electron micrograph of a process extending into the lumen of the frontal organ of Stage I[14] larva of *Hyla regilla*, fixed in 1% OsO_4, pH 7.3, 0°C for 2 hrs. and embedded in Epon; sections stained overnight with 2% uranyl acetate. *d*, discs; *is*, inner segment; *l*, lumen; *m*, mitochondria; *os*, outer segment; *s*, matrix of outer segment; *v*, vesicles. Arrows show infoldings of cell membrane to form the discs. × 32,000.

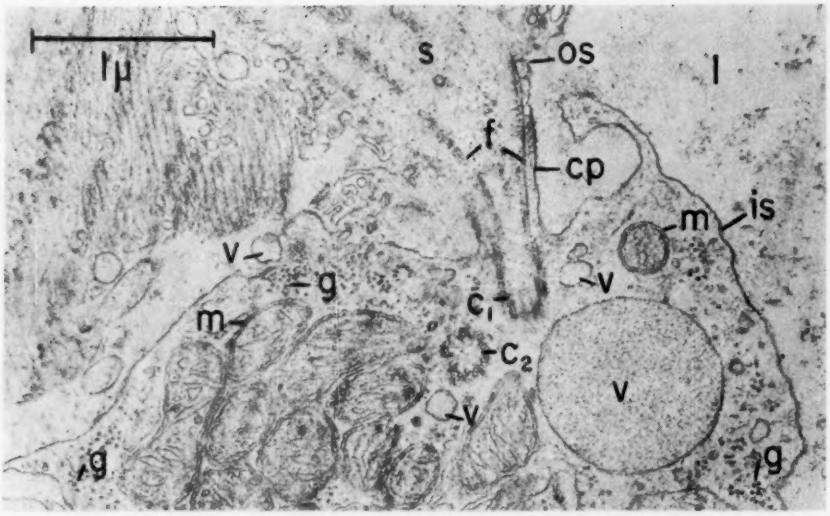

FIG. 2.—Electron micrograph of the connecting piece and parts of the inner and outer segments of another process from the same specimen as that shown in Fig. 1. c_1, axial centriole; c_2, oblique centriole; *cp*, connecting piece; *f*, fibrils; *g*, granules; *is*, inner segment; *l*, lumen; *m*, mitochondria; *os*, outer segment; *s*, matrix of outer segment; *v*, vesicles. × 28,000.

variation in the blanching reaction of normal larvae I selected for this study only animals which gave a good response when placed in the dark. Repeated tests for blanching were conducted, but I was unable to establish a clear difference between the experimental and control animals. Both types paled when kept for two hours in a light-proof constant-temperature cabinet set at 18°C. Although all tadpoles had given a strong blanching reaction before surgery, now some in both groups showed a fair response only, the number varying slightly from test to test.

On the assumption that the dominant role of the lateral eyes might obscure any photoreceptive activity of the frontal organ, I removed the eyes from the stirnorganectomized and sham-operated animals in lots of twenty (10 experimental and 10 control larvae) selected at random. Within the next ten days the animals were tested four or five times for the blanching reaction. Each group of ten larvae was kept in a pan and the eight groups were maintained under the same conditions of lighting, temperature, and food as far as possible. To test a given lot, the experimental and control tadpoles were transferred to two clean pans of pond water and placed in the dark at 18°C for two hours. Each animal was then examined under a dissecting microscope and graded on a 1 (maximal blanching) to 5 (no blanching) scale, similar to that used by Hogben and Slome.[16]

The removal of the eyes greatly reduced the average blanching reaction in both groups. The difference between stirnorganectomized and sham-operated animals, however, was not marked; yet I believe it was significant. The controls gave on the average a stronger response although some in each lot showed no pigmentary shift (grade 5 all over the body). On the other hand a few experimental larvae gave a good (grade 2) or even occasionally an excellent (grade 1) reaction, in which all melanophores on the body, exclusive of the tail, were punctate.

I did not consider a statistical analysis appropriate. In the first place, the grades of pallor could not be sharply defined. Secondly, there was usually considerable variation in the degree of pigmentary shift in the melanophores of an individual tadpole. I attempted, therefore, to assign an over-all rating to each animal on each test. The average scores based upon four separate tests of each lot are as follows. *Controls:* 30 per cent strong reaction (grades 1–2), 20 per cent fair response (grade 3), 50 per cent reaction poor or absent (grades 4–5). *Experimentals:* 10, 10, and 80 per cent, respectively. The asserted difference does not rest, however, upon the above observations exclusively. More convincing was the localized response of the melanophores in the epiphysial region of the controls. More often than not these cells exhibited a greater concentration of pigment than those in other parts of the head. Frequently a sham-operated animal scored as grade 4 or 5 on the basis of general appearance, exhibited grades 3–1 around the frontal organ and above the epiphysis. Such a localized response was not observed in the stirnorganectomized animals.

Discussion.—Earlier workers[1, 4] assumed on the basis of an examination of several species of hylid frogs that the frontal organ was absent in the genus *Hyla*, an exception among anuran amphibians. Winterhalter,[17] however, found it in larvae of *H. arborea*, and it is readily observed through the clear epidermis of tadpoles of *H. regilla* up to the stage (approximately XVI[14]) at which chromatophores become so numerous in the outer layer of the integument that the organ is obscured, and it may be revealed in later stages of metamorphosis and in the completely transformed young treefrog by simply reflecting the overlying skin.

Evidence in support of a photoreceptive function of the frontal organ consists of the following. First, the organ is favorably situated for receiving light: dorsal to the heavy curtain of dermal and meningeal melanophores and beneath a clear epidermis which in some frogs retains its transparency in the adult in a localized region, the brow spot, directly above the frontal organ.[1, 3] Second, there is a striking similarity in fine structure between the processes of known photoreceptors and those of the stirnorgan, described and figured here. Third, stirnorganectomy combined with enucleation of the eyes in young tadpoles of *H. regilla* (see above) eliminated the blanching reaction in many instances and greatly reduced it in most others. A few larvae, however, gave a strong response which might be explained by the presence in these specimens of active photoreceptors in the epiphysis or brain. Eakin and Westfall[18] have observed laminated processes in electron micrographs of the epiphysis of the fence lizard, *Sceloporus occidentalis*, and Steyn[19] has reported similar structures in that of *Cordylus polyzonus*. Moreover, light microscopy has shown a similarity in the processes of the amphibian epiphysis[2, 13, 20] and those of the stirnorgan.[2, 3, 13] More difficult of explanation, on the other hand, are control specimens in my experiment in which no blanching whatsoever was observed despite an undisturbed frontal organ. Lastly, there is increasing evidence that the reptilian homologue of the stirnorgan, the parietal eye, is indeed a light-sensitive structure.[9, 21–23]

Summary.—The fine structure of processes extending into the lumen of the frontal organ of young tadpoles of the Pacific Treefrog, *Hyla regilla*, is described and illustrated with electron micrographs. The processes are similar to rods and cones of the vertebrate lateral eye. Larvae without frontal organ and eyes give on the

average a weaker blanching reaction (concentration of pigment in the dermal melanophores of the body) when the tadpoles are placed in the dark than do the controls without eyes. It is concluded that the cells which bear the processes are photoreceptors.

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ADDENDUM: THE SCHOENFLIES THEOREM FOR POLYHEDRA

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The differentiability properties in the theorem of the note in question[1] are established with the aid of certain approximating equipotential manifolds mentioned in the outlined proof of Lemma 4. They depend on such manifolds being diffeomorphic to a euclidean sphere. The latter property is presently proved with the aid of a theorem of Thom[2] to the effect that the differentiable structure on the n-ball is unique if the *Hauptvermutung* is valid for the n-ball. The author hopes to complete a proof independent of Thom's theorem and of any *Hauptvermutung* hypothesis. Note also that Lemmas 1–4 are true only with certain upper bounds on $(\delta_0, \ldots, \delta_n)$ in addition to those already specified.

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Vestine, Ernest Harry, 1954 (13), Rand Corporation, 1700 Main Street, Santa Monica, California

Vickery, Hubert **B**radford, 1943 (14), Connecticut Agricultural Experiment Station, New Haven 4, Connecticut

Villard, Oswald Garrison, **Jr.,** 1958 (13), Radioscience Laboratory, Stanford University, Stanford, California

Visscher, Maurice Bolks, 1956 (9), Department of Physiology, University of Minnesota, Minneapolis 14, Minnesota

von **Békésy, Georg,** 1956 (12), Psycho-Acoustic Laboratory, Memorial Hall, Harvard University, Cambridge 38, Massachusetts

von Kármán, Theodore, 1938 (4), 1501 South Marengo Avenue, Pasadena 5, California

Waksman, Selman Abraham, 1942 (10), Institute of Microbiology, Rutgers, The State University, New Brunswick, New Jersey

Wald, George, 1950 (14), The Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge 38, Massachusetts

Walker, John Charles, 1945 (7), 206 Horticulture Building, University of Wisconsin, Madison 6, Wisconsin

Wall, Frederick Theodore, 1961 (5), Department of Chemistry and Chemical Engineering, University of Illinois, Urbana, Illinois

Walsh, Joseph Leonard, 1936 (1), Widener 474, Harvard University, Cambridge 38, Massachusetts

Warner, John Christian, 1956 (5), Carnegie Institute of Technology, Pittsburgh 13, Pennsylvania

Watson, Cecil James, 1959 (10), Department of Medicine, University of Minnesota Hospital, Minneapolis 14, Minnesota

Webster, David Locke, 1923 (3), 1830 Cowper Street, Palo Alto, California

Weinberg, Alvin Martin, 1961 (3), Oak Ridge National Laboratory, P. O. Box X, Oak Ridge, Tennessee

Weiss, Paul Alfred, 1947 (8), Rockefeller Institute, New York 21, New York

Weisskopf, Victor Frederick, 1952 (3), Department of Physics, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Went, Frits Warmolt, 1947 (7), Missouri Botanical Garden, 2315 Tower Grove Avenue, St. Louis 10, Missouri

Wentzel, Gregor, 1959 (3), The Enrico Fermi Institute for Nuclear Studies, University of Chicago, Chicago 37, Illinois

Werkman, Chester Hamlin, 1946 (14), Department of Bacteriology, Iowa State University, Ames, Iowa

Westheimer, Frank Henry, 1954 (5), Department of Chemistry, Harvard University, 12 Oxford Street, Cambridge 38, Massachusetts

Wetmore, Alexander, 1945 (8), Smithsonian Institution, Washington 25, D. C.

Wetmore, Ralph Hartley, 1954 (7), The Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge 38, Massachusetts

Wever, Ernest Glen, 1940 (12), 110 Eno Hall, Princeton University, Princeton, New Jersey

Wheeler, John Archibald, 1952 (3), Palmer Physical Laboratory, Princeton University, Princeton, New Jersey

Whipple, Fred Lawrence, 1959 (2), Astrophysical Observatory, Smithsonian Institution, 60 Garden Street, Cambridge 38, Massachusetts

Whipple, George Hoyt, 1929 (10), School of Medicine and Dentistry, University of Rochester, 260 Crittenden Boulevard, Rochester 20, New York

Whitford, Albert Edward, 1954 (2), Lick Observatory, Mount Hamilton, California

Whitney, Hassler, 1945 (1), The Institute for Advanced Study, Princeton, New Jersey

Whyburn, Gordon Thomas, 1951 (1), Department of Mathematics, University of Virginia, Charlottesville, Virginia

Wiesner, Jerome Bert, 1960 (4), President's Science Advisory Committee, Room 200, Executive Office Building, Washington 25, D. C.

Wiggers, Carl John, 1951 (9), Frank E. Bunts Educational Institute of Cleveland Clinic, 1360 Cleveland Heights Boulevard, Cleveland Heights 21, Ohio

Wigner, Eugene Paul, 1945 (3), Fine Hall, Princeton University, Princeton, New Jersey

Willey, Gordon Randolph, 1960 (11), Peabody Museum, Harvard University, Cambridge 38, Massachusetts

Williams, Carroll Milton, 1960 (8), The Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge 38, Massachusetts

Williams, Howel, 1950 (6), Department of Geology, University of California, Berkeley 4, California

Williams, John Harry, 1961 (3), School of Physics, University of Minnesota, Minneapolis 14, Minnesota

Williams, John Warren, 1952 (5), Department of Chemistry, University of Wisconsin, Madison 6, Wisconsin

Williams, Robert R., 1945 (5), 45 Woodland Avenue, Summit, New Jersey

Williams, Robley Cook, 1955 (14), Virus Laboratory, University of California, Berkeley 4, California

Williams, Roger John, 1946 (5), Clayton Foundation Biochemical Institute, University of Texas, Austin 12, Texas

Willier, Benjamin Harrison, 1945 (8), Department of Biology, Johns Hopkins University, Baltimore 18, Maryland

Wilson, David Wright, 1955 (14), Department of Biochemistry, School of Medicine, University of Pennsylvania, Philadelphia 4, Pennsylvania

Wilson, Edgar Bright, Jr., 1947 (5), Department of Chemistry, Harvard University, 12 Oxford Street, Cambridge 38, Massachusetts

Wilson, Edwin Bidwell, 1919 (3), 42 Brington Road, Brookline 46, Massachusetts

Wilson, Olin Chaddock, 1960 (2), Mount Wilson and Palomar Observatories, 813 Santa Barbara Street, Pasadena, California

Wilson, Perry William, 1955 (7), Department of Bacteriology, University of Wisconsin, Madison 6, Wisconsin

Wilson, Robert Erastus, 1947 (4), United States Atomic Energy Commission, Washington 25, D. C.

Wilson, Robert Rathbun, 1957 (3), Laboratory of Nuclear Studies, Cornell University, Ithaca, New York

Winstein, Saul, 1955 (5), Department of Chemistry, University of California, Los Angeles 24, California

Wintersteiner, Oskar, 1950 (14), Squibb Institute for Medical Research, New Brunswick, New Jersey

Wolfrom, Melville Lawrence, 1950 (5), Department of Chemistry, The Ohio State University, 121 West 19th Avenue, Columbus 10, Ohio

Wood, Harland Goff, 1953 (14), Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland 6, Ohio

Wood, William Barry, Jr., 1959 (10), School of Medicine, Johns Hopkins University, 725 North Wolfe Street, Baltimore 5, Maryland

Woodring, Wendell Phillips, 1946 (6), United States Geological Survey, Washington 25, D. C.

Woodward, Robert Burns, 1953 (5), Department of Chemistry, Harvard University, 12 Oxford Street, Cambridge 38, Massachusetts

Woodworth, Robert Sessions, 1921 (12), 400 West 119th Street, New York 27, New York

Woolley, Dilworth Wayne, 1952 (14), Rockefeller Institute, New York 21, New York

Woolsey, Clinton Nathan, 1960 (9), Laboratory of Neurophysiology, 283 Medical Sciences Building, University of Wisconsin, Madison 6, Wisconsin

Wright, Sewall Green, 1934 (8), Department of Genetics, University of Wisconsin, Madison 6, Wisconsin

Wu, Chien-Shiung, 1958 (3), Department of Physics, Columbia University, New York 27, New York

Wulf, Oliver Reynolds, 1949 (13), United States Weather Bureau, Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena 4, California

Wyckoff, Ralph Walter Graystone, 1949 (5), Departments of Bacteriology and Physics, University of Arizona, Tucson 25, Arizona

Yoder, Hatten Schuyler, **Jr.,** 1958 (6), Geophysical Laboratory, Carnegie Institution of Washington, 2801 Upton Street, N. W., Washington 8, D. C.

Yost, Don Merlin Lee, 1944 (5), California Institute of Technology, Pasadena 4, California

Young, William Gould, 1951 (5), Department of Chemistry, University of California, Los Angeles 24, California

Zacharias, Jerrold Reinach, 1957 (3), Department of Physics, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Zachariasen, Frederik **William H**oulder, 1949 (3), Division of the Physical Sciences, University of Chicago, Chicago 37, Illinois

Zariski, Oscar, 1944 (1), Department of Mathematics, Harvard University, 2 Divinity Avenue, Cambridge 38, Massachusetts

Zener, Clarence Melvin, 1959 (4), Westinghouse Research Laboratories, Beulah Road, Churchill Borough, Pittsburgh 35, Pennsylvania

Zimm, Bruno Hasbrouck, 1958 (5), School of Science and Engineering, University of California, La Jolla, California

Zinn, Walter **H**enry, 1956 (4), Combustion Engineering, Inc., Nuclear Division, Windsor, Connecticut

Zirkle, Raymond Elliott, 1959 (8), Committee on Biophysics, University of Chicago, 5640 Ellis Avenue, Chicago 37, Illinois

Zworykin, Vladimir **K**osma, 1943 (4), RCA Laboratories, David Sarnoff Research Center, Princeton, New Jersey

Zygmund, Antoni, 1960 (1), Department of Mathematics, University of Chicago, Chicago 37, Illinois

Number of Members July 1, 1961: 631.

MEMBERS EMERITI

Albright, Fuller, 1952 (10), 271 Goddard Avenue, Brookline 46, Massachusetts

Bailey, Irving **W**idmer, 1929 (7), Harvard University Herbarium, 22 Divinity Avenue, Cambridge 38, Massachusetts

Coble, Arthur Byron, 1924 (1), Lykens Hotel, Lykens, Pennsylvania

Cole, Rufus, 1922 (10), Mt. Kisco, New York

Coolidge, William **D**avid, 1925 (3), 1480 Lenox Road, Schenectady 8, New York

Hartman, Carl Gottfried, 1937 (8), 219 Norwood Avenue, North Plainfield, New Jersey

Kelley, Walter Pearson, 1943 (6), 140 Giannini Hall, University of California, Berkeley 4, California

Kidder, Alfred Vincent, 1936 (11), 41 Holden Street, Cambridge 38, Massachusetts

Saunders, Frederick Albert, 1925 (3), South Hadley, Massachusetts

Schultz, Adolph Hans, 1939 (11), Anthropologisches Institut, Sempersteig 3, Zurich, Switzerland

Tyzzer, Ernest Edward, 1942 (10), 175 Water Street, Wakefield, Massachusetts

Vandiver, Harry Shultz, 1934 (1), Box 7881, University of Texas, Austin 12, Texas

Number of Members Emeriti July 1, 1961: 12.

FOREIGN ASSOCIATES

The number in parentheses following the year of election indicates association within the Sections of the National Academy of Sciences.

Adrian of **Cambridge,** Edgar Douglas, Baron, 1941 (9), Trinity College, Cambridge, England

Alexandroff, Paul A., 1947 (1), Mathematical Institute of the Academy of Sciences of the U. S. S. R., Bolshaya Kalushskaya 19, Moscow, U. S. S. R.

Ambartsumian, Victor Amazaspovich, 1959 (2), Burakan Astronomical Observatory, Erevan, Armenia, U.S.S.R.

Bailey, Sir Edward, 1944 (6), 76 Hampstead Way, London, N. W. 11, England

Bartlett, Sir Frederic Charles, 1947 (12), 161 Huntingdon Road, Cambridge, England

Best, Charles Herbert, 1950 (9), Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada

Bohr, Niels, 1925 (3), Institute for Theoretical Physics, Blegdamsvej 15, Copenhagen, Denmark

Born, Max, 1955 (3), Marcard Strasse 4, Bad Pyrmont, Germany

Bragg, Sir William **Lawrence,** 1945 (3), The Royal Institution, 21 Albemarle Street, London, W. 1, England

de Broglie, Prince Louis, 1948 (3), 94 Rue Perronet, Neuilly-sur-Seine, France

Brun, Edmond Antoine, 1960 (4), University of Paris, 8–10, place du Commerce, Paris XV, France

Bullard, Sir Edward Crisp, 1959 (13), Madingley Rise, Madingley Road, Cambridge, England

Bullen, Keith Edward, 1961 (13), Department of Applied Mathematics, University of Sydney, Sydney, Australia

Burnet, Sir Macfarlane, 1954 (10), The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

Caso, Alfonso, 1943 (11), Avenida Central 234, Tlacopac, Villa Obregón, Mexico 20, D. F.

Chapman, Sydney, 1946 (13), High Altitude Observatory, Boulder, Colorado, U. S. A.

Dale, Sir Henry Hallett, 1940 (9), The Wellcome Trust, 52 Queen Anne Street, London, W. 1, England

Dirac, Paul Adrien Maurice, 1949 (1), Department of Mathematics, St. John's College, Cambridge, England

Ephrussi, Boris, 1961 (7), Laboratoire de Génétique Physiologique du C.N.R.S., Gif sur Yvette (Seine et Oise), France

Eskola, Pentti Eelis, 1951 (6), Helsinki University, Snellmanink. 5, Helsinki, Finland

Fisher, Sir Ronald Aylmer, 1948 (8), Division of Statistics, C.S.I.R.O., University of Adelaide, Adelaide, South Australia

von Frisch, Karl, 1951 (8), The Zoological Institute, University of Munich, Munich, Germany

Geijer, Per, 1958 (6), Agnevaegen 5, Djursholm I, Sweden

Gregory, Frederick Gugenheim, 1956 (7), Imperial College of Science and Technology, London, S.W. 7, England

Hadamard, Jacques, 1926 (1), 12 Rue Emile Faguet, Paris XIV, France

Hartmann, Max, 1959 (8), Buchenbühl, Post Weiler im Allgäu, West Germany

Heisenberg, Werner, 1961 (3), Max Planck Institut für Physik und Astrophysik, Aumeisterstrasse 6, Munich 23, Germany

Hill, Archibald Vivian, 1941 (9), 16 Bishopswood Road, Highgate, London, N.6, England

Hinshelwood, Sir Cyril Norman, 1960 (5), Department of Chemistry, Exeter College, Oxford, England

Hodge, William Vallance Douglas, 1959 (1), The Master's Lodge, Pembroke College, Cambridge, England

Hopf, Heinz, 1957 (1), Swiss Federal Institute of Technology, Zurich, Switzerland

Houssay, Bernardo Alberto, 1940 (9), Viamonte 2790, Buenos Aires, Argentina

Jeffreys, Sir Harold, 1945 (13), St. John's College, Cambridge, England

Kapitza, Peter Leonidovich, 1946 (3), S. I. Vavilov Institute of Physical Problems, Academy of Sciences of the U.S.S.R., Moscow, U.S.S.R.

Karrer, Paul, 1945 (5), University of Zurich, Zurich, Switzerland

Kihara, Hitoshi, 1958 (7), National Institute of Genetics, Misima, Japan

Landau, Lev Davidovich, 1960 (3), S. I. Vavilov Institute of Physical Problems, Academy of Sciences of the U.S.S.R., Moscow, U.S.S.R.

Leloir, Luis F., 1960 (14), Instituto de Investigaciones Bioquimicas, Obligado 2490, Buenos Aires, Argentina

Levi, Giuseppe, 1940 (8), Instituto di Anatomia Umana, Corso Massimo D'Azeglio, 52, Turin, Italy

Lim, Robert K. S., 1942 (9), Medical Sciences Research Laboratory, Miles Laboratories, Inc., Elkhart, Indiana, U. S. A.

Lindblad, Bertil, 1955 (2), Stockholm Observatory, Saltsjöbaden, Sweden

Lwoff, André, 1955 (9), Institut Pasteur, Paris XV, France

Michotte, Albert Edouard (Baron Michotte van den Berck), 1956 (12), University of Louvain, Louvain, Belgium

Mott, Nevill Francis, 1957 (3), University of Cambridge, Cavendish Laboratory, Free School Lane, Cambridge, England

Oort, Jan Hendrik, 1952 (2), Observatory of Leiden, Leiden, The Netherlands

Penfield, Wilder, 1953 (10), Montreal Neurological Institute, 3801 University Street, Montreal 2, Canada

Pérès, Joseph Jean Camille, 1956 (1), University of Paris, Paris V, France

Piéron, Henri, 1949 (12), Institute of Psychology, University of Paris, Paris, France

Portevin, Albert M. G. R., 1954 (4), 21, Boulevard de Beauséjour, Paris XVI, France

Prelog, Vladimir, 1961 (5), Laboratorium für organische Chemie, Eidgenossische Technische Hochschule, Zurich, Switzerland

Reichstein, Tadeus, 1952 (5), Organisch-chemische Anstalt, St. Johanns-Ring 19, Basel, Switzerland

Robinson, Sir Robert, 1934 (5), 170 Piccadilly, London, W. 1, England

Ruzicka, Leopold, 1944 (5), Department of Organic Chemistry, Institute of Technology, Zurich, Switzerland

Southwell, Sir Richard Vynne, 1943 (4), The Old House, Trumpington, Cambridge, England

Steacie, Edgar William Richard, 1957 (5), National Research Council, Ottawa, Canada

Svedberg, The, 1945 (5), Fysikalisk-Kemiska Institutionen, Uppsala University, Uppsala, Sweden

Taylor, Sir Geoffrey Ingram, 1945 (1), Trinity College, Cambridge, England

Theorell, Axel **Hugo,** 1957 (14), Nobel Institute of Medicine, Solnavagen 1, Stockholm 60, Sweden

Tiselius, Arne W. K., 1949 (14), Institute of Biochemistry, Uppsala University, Uppsala, Sweden

Todd, Sir Alexander Robertus, 1955 (5), University of Cambridge, University Chemical Laboratory, Pembroke Street, Cambridge, England

Vallée-Poussin, C. de la, 1929 (1), 42, Avenue du Houx, Boitsfort, Belgium

Vening Meinesz, Felix **A**ndries, 1939 (13), Potgieterlaan 5, Amersfoort, The Netherlands

Watson, D. M. S., 1938 (8), University College, Gower Street, London, W.C. 1, England

Winge, Öjvind, 1949 (14), Department of Physiology, Carlsberg Laboratory, Copenhagen (Valby), Denmark

Yukawa, Hideki, 1949 (3), Yukawa Hall, Kyoto University, Kyoto, Japan

Number of Foreign Associates July 1, 1961: 65.

SECTIONS

(1) *Mathematics*—36 members

Whitney, Hassler, *Chairman* (1964)
Ahlfors, L. V.
Albert, A. A.
Alexander, J. W.
Bochner, S.
Brauer, R. D.
Chern, S. S.
Courant, R.
Doob, J. L.
Douglas, Jesse
Eilenberg, S.
Eisenhart, L. P.
Evans, G. C.
Feller, William
Friedrichs, K. O.
Gödel, Kurt
Hille, Einar
Jacobson, Nathan
Lefschetz, Solomon
Mac Lane, Saunders
McShane, E. J.
Montgomery, Deane
Moore, R. L.
Morse, Marston
Murnaghan, F. D.
Shannon, C. E.
Smith, Paul A.
Spencer, D. C.
Steenrod, N. E.
Stone, M. H.
Thomas, T. Y.
Tukey, John W.
Walsh, J. L.
Whyburn, G. T.
Zariski, O.
Zygmund, Antoni

Foreign Associates

Alexandroff, P. A.
Dirac, P. A. M.
Hadamard, Jacques
Hodge, W. V. D.
Hopf, Heinz
Pérès, Joseph
Taylor, Sir Geoffrey
Vallée-Poussin, C. de la

(2) ***Astronomy*—30 members**

Mayall, N. U., *Chairman* (1962)
Abbot, C. G.
Babcock, H. D.
Babcock, H. W.
Bowen, I. S.
Brouwer, Dirk
Chandrasekhar, S.
Clemence, G. M.
Goldberg, Leo
Greenstein, J. L.
Joy, A. H.
Kuiper, G. P.
McMath, R. R.
Menzel, D. H.
Merrill, P. W.
Minkowski, R. L.
Morgan, W. W.
Nicholson, S. B.
Schwarzschild, M.
Seares, F. H.
Shane, C. D.
Shapley, Harlow
Slipher, V. M.
Spitzer, Lyman, Jr.
Stebbins, Joel
Struve, Otto
Tousey, Richard
Whipple, F. L.
Whitford, A. E.
Wilson, Olin C.

Foreign Associates

Ambartsumian, V.
Lindblad, Bertil
Oort, Jan Hendrik

(3) ***Physics*—96 members**

Allison, S. K., *Chairman* (1963)
Alvarez, L. W.
Anderson, C. D.
Anderson, H. L.
Bacher, R. F.
Bainbridge, K. T.
Bardeen, John
Beams, J. W.
Bethe, H. J.
Birge, R. T.
Bleakney, Walker
Bloch, Felix
Bloembergen, N.
Bonner, T. W.
Bradbury, N. E.
Brattain, W. H.
Breit, Gregory
Bridgman, P. W.
Brillouin, Leon
Brode, R. B.
Chamberlain, Owen
Coblentz, W. W.
Compton, A. H.
Condon, E. U.
Dennison, D. M.
Deutsch, M.
DuBridge, L. A.
DuMond, J. W. M.
Dunning, J. R.
Elsasser, W. M.
Epstein, P. S.
Feynman, R. P.
Fowler, W. A.
Franck, James
Gamow, George
Gell-Mann, Murray
Goldhaber, M.
Goudsmit, S. A.
Herb, R. G.
Herzfeld, K. F.
Hofstadter, R.
Houston, W. V.
Hull, A. W.
Inghram, Mark G.
Kemble, E. C.
Kerst, D. W.
Kittel, C.
Kusch, P.
Lamb, W. E., Jr.
Land, E. H.
Lauritsen, C. C.
Loomis, F. W.
Marshak, R. E.
Mayer, M. G.
McMillan, E. M.
Meggers, W. F.
Morse, P. M.
Mulliken, R. S.
Nier, A. O. C.
O'Brien, Brian
Oppenheimer, J. R.
Panofsky, W. K. H.
Pound, R. V.
Purcell, E. M.
Rabi, I. I.
Ramsey, N. F.
Robertson, H. P.
Rossi, Bruno
Schiff, L. I.
Schwinger, Julian
Segrè, E.
Seitz, Frederick
Serber, R.
Simpson, J. A.
Slater, J. C.
Stern, Otto
Street, J. C.
Szilard, Leo
Teller, Edward
Thomas, L. H.

Townes, C. H.
Tuve, M. A.
Uhlenbeck, G. E.
Van Vleck, J. H.
Webster, D. L.
Weinberg, Alvin M.
Weisskopf, V. F.
Wentzel, Gregor
Wheeler, J. A.
Wigner, E. P.
Williams, John H.
Wilson, Edwin B.
Wilson, Robert R.
Wu, C. S.
Zacharias, J. R.
Zachariasen, W. H.

Foreign Associates

Bohr, Niels
Born, Max
Bragg, Sir Lawrence
de Broglie, Prince Louis
Heisenberg, Werner
Kapitza, P. L.
Landau, L. D.
Mott, N. F.
Yukawa, Hideki

(4) *Engineering*—47 members

Fisk, J. B., *Chairman* (1962)
Astin, A. V.
Bain, E. C.
Benedict, Manson
Bode, H. W.
Briggs, L. J.
Bush, Vannevar
Chipman, John
Curme, G. O., Jr.
Darken, L. S.
Den Hartog, J. P.
Draper, C. S.
Dryden, H. L.
Fletcher, Harvey
Foote, P. D.
Gibbs, W. F.
Gilliland, E. R.
Greenewalt, C. H.
Hoover, Herbert
Hunsaker, J. C.
Jeffries, Zay
Kelly, M. J.
Kinzel, A. B.
Lewis, W. K.
Loomis, A. L.
Mehl, R. F.
Murphree, E. V.
Olson, Harry F.
Pierce, J. R.
Raymond, A. E.
Savage, J. L.
Sherwood, T. K.
Shockley, W.
Slepian, Joseph
Smith, C. S.
Soderberg, C. R.
Stratton, J. A.
Suits, C. G.
Terman, F. E.
Thomas, C. A.
Timoshenko, Stephen
von Kármán, T.
Wiesner, J. B.
Wilson, Robert E.
Zener, Clarence
Zinn, W. H.
Zworykin, V. K.

Foreign Associates

Brun, Edmond A.
Portevin, Albert M. G. R.
Southwell, Sir Richard V.

(5) *Chemistry*—95 members

Pitzer, K. S., *Chairman* (1962)
Adams, Roger
Badger, R. M.
Baker, W. O.
Bartlett, P. D.
Blomquist, A. T.
Bolton, E. K.
Brewer, Leo
Brode, W. R.
Brown, H. C.
Calvin, Melvin
Conant, J. B.
Cope, A. C.
Cram, Donald J.
Crawford, Bryce, Jr.
Daniels, Farrington
Davidson, N.
Debye, Peter
Djerassi, Carl
Doering, William
Doty, Paul
Elderfield, R. C.
Emmett, P. H.
Eyring, Henry
Ferry, John D.
Fieser, L. F.
Flory, P. J.
Folkers, Karl
Fuoss, R. M.
Fuson, R. C.
Gates, M.
Giauque, W. F.
Gilman, Henry
Gutowsky, H. S.
Hammett, L. P.
Harned, H. S.
Hauser, C. R.
Hendricks, S. B.
Hildebrand, J. H.
Hirschfelder, J. O.
Hornig, D. F.
Jacobs, W. A.
Johnson, J. R.
Johnson, W. S.

Keyes, F. G.
Kimball, G. E.
Kistiakowsky, G. B.
Kolthoff, I. M.
Kraus, C. A.
La Mer, V. K.
Leonard, N. J.
Libby, W. F.
Lind, S. C.
Lipscomb, W. N.
Longsworth, L. G.
Lucas, H. J.
MacInnes, D. A.
Mark, H. F.
Marvel, C. S.
Mayer, J. E.
McElvain, S. M.
Newman, M. S.
Niemann, C. G.
Noyes, W. A., Jr.
Onsager, Lars
Pauling, Linus
Roberts, John D.
Rossini, F. D.
Scatchard, George
Seaborg, G. T.
Shedlovsky, Theodore
Sheehan, J. C.
Smith, L. I.
Smyth, C. P.
Spedding, F. H.
Stockmayer, W. H.
Stork, Gilbert
Tarbell, D. S.
Taube, Henry
Tishler, Max
Urey, H. C.
Wall, Frederick T.
Warner, J. C.
Westheimer, F. H.
Williams, J. W.
Williams, Robert R.
Williams, Roger J.
Wilson, E. Bright, Jr.
Winstein, Saul
Wolfrom, M. L.
Woodward, R. B.
Wyckoff, R. W. G.
Yost, D. M.
Young, W. G.
Zimm, B. H.

Foreign Associates

Hinshelwood, Sir C. N.
Karrer, Paul
Prelog, Vladimir
Reichstein, Tadeus
Robinson, Sir Robert
Ruzicka, Leopold
Steacie, E. W. R.
Svedberg, The
Todd, Sir Alexander

(6) *Geology*—33 members

Hess, H. H., *Chairman* (1963)
Abelson, P. H.
Allen, E. T.
Anderson, C. A.
Birch, Francis
Blackwelder, Eliot
Bradley, W. H.
Bramlette, M. N.
Bucher, W. H.
Buddington, A. F.
Buerger, M. J.
Chaney, R. W.
Cloos, Ernst
Cloud, Preston E., Jr.
Dunbar, C. O.
Gilluly, James
Gregory, W. K.
Hedberg, H. D.
Hewett, D. F.
Hubbert, M. King
Knopf, Adolph
Krauskopf, K. B.
Longwell, C. R.
Lovering, T. S.
Nolan, T. B.
Rubey, W. W.
Russell, R. J.
Schairer, J. F.
Simpson, G. G.
Turner, F. J.
Williams, Howel
Woodring, W. P.
Yoder, H. S., Jr.

Foreign Associates

Bailey, Sir Edward
Eskola, Pentti
Geijer, Per

(7) *Botany*—47 members

Riker, A. J., *Chairman* (1962)
Anderson, Edgar
Arnon, Daniel I.
Barker, H. A.
Beadle, G. W.
Benzer, Seymour
Blinks, L. R.
Bonner, David M.
Bonner, James
Borthwick, H. A.
Braun, A. C.
Brink, R. A.
Burkholder, P. R.
Burris, R. H.
Chandler, W. H.
Clausen, Jens
Cleland, R. E.
Couch, J. N.
Delbrück, Max
Esau, Katherine
Fred, E. B.
Goddard, D. R.
Hershey, A. D.

Hollaender, A.
Horsfall, J. G.
Jones, D. F.
Kaufmann, B. P.
Lederberg, J.
Luria, S. E.
Mangelsdorf, P. C.
McClintock, Barbara
Osterhout, W. J. V
Raper, K. B.
Rhoades, M. M.
Robbins, W. J.
Roberts, R. B.
Sax, Karl
Sinnott, E. W.
Skoog, Folke
Stakman, E. C.
Stebbins, G. L.
Thimann, K. V.
Van Niel, C. B.
Walker, J. C.
Went, F. W.
Wetmore, R. H.
Wilson, P. W.

Foreign Associates

Ephrussi, Boris
Gregory, F. G.
Kihara, H.

(8) *Zoology and Anatomy*—56 members

Mayr, Ernst, *Chairman* (1964)
Bartelmez, G. W.
Bigelow, H. B.
Bloom, William
Bodenstein, D. H.
Bodian, David
Burns, R. K.
Castle, W. E.
Cleveland, L. R.
Colbert, E. H.
Corner, G. W.
Crow, James F.
Danforth, C. H.
Demerec, Milislav
Dobzhansky, Th.
Dunn, L. C.
Glass, Bentley
Griffin, D. R.
Hamburger, Viktor
Haskins, C. P.
Hisaw, F. L.
Holtfreter, J.
Hubbs, C. L.
Hutchinson, G. E.
Hyman, Libbie
Irwin, M. R.
Jacobs, M. H.
Kellogg, Remington
Lerner, I. M.
Lewis, W. H.
Mazia, Daniel
Metz, C. W.
Miller, A. H.
Mirsky, A. E.
Muller, H. J.
Nicholas, J. S.
Painter, T. S.
Palade, G. E.
Petrunkevitch, A.
Riddle, Oscar
Romer, A. S.
Schmitt, F. O.
Scholander, Per F.
Schrader, Franz
Smith, Philip E.
Sonneborn, T. M.
Stern, Curt
Stone, W. S.
Sturtevant, A. H.
Twitty, V. C.
Weiss, Paul
Wetmore, Alexander
Williams, C. M.
Willier, B. H.
Wright, Sewall
Zirkle, R. E.

Foreign Associates

Fisher, Sir Ronald
von Frisch, Karl
Hartmann, Max
Levi, Giuseppe
Watson, D. M. S.

(9) *Physiology*—35 members

Long, C. N. H., *Chairman* (1963)
Astwood, E. B.
Aub, Joseph C.
Bard, Philip
Brink, Frank, Jr.
Bronk, Detlev W.
Cole, K. S.
Comroe, Julius H., Jr.
Cournand, André
Davis, Hallowell
Erlanger, Joseph
Evans, H. M.
Fenn, W. O.
Forbes, Alexander
Gasser, H. S.
Gerard, R. W.
Hartline, H. K.
Hastings, A. B.
Landis, E. M.
Lloyd, D. P. C.
Loeb, R. F.
Lorente de Nó, R.
Magoun, H. W.
Mann, Frank
Marshall, E. K., Jr.
Meek, W. J.
Pitts, R. F.
Redfield, A. C.
Richards, A. N.
Richards, D. W.
Schmidt, C. F.
Smith, Homer W.
Visscher, M. B.
Wiggers, C. J.
Woolsey, C. N.

Foreign Associates

Lord Adrian
Best, C. H.
Dale, Sir Henry
Hill, A. V.
Houssay, B. A.
Lim, Robert K. S.
Lwoff, André

(10) *Pathology and Microbiology*—43 members

Shope, R. E., *Chairman* (1963)
Armstrong, Charles
Bailey, P.
Blalock, Alfred
Cannon, P. R.
Castle, W. B.
Coggeshall, L. T.
Dalldorf, Gilbert
Dingle, J. H.
Dochez, A. R.
Dragstedt, L. R.
Dubos, R. J.
Dulbecco, Renato
Enders, J. F.
Francis, Thomas, Jr.
Goebel, W. F.
Heidelberger, Michael
Horsfall, F. L., Jr.
Hotchkiss, R. D.
Huebner, R. J.
Huggins, C. B.
Little, C. C.
Long, E. R.
MacLeod, C. M.
Maxcy, K. F.
McMaster, P. D.
Meyer, K. F.
Miller, C. P.
Opie, E. L.
Paul, John R.
Puck, T. T.
Rich, A. R.
Rivers, T. M.
Robertson, O. H.
Rous, Peyton
Sabin, Albert B.
Smadel, J. E.
Taliaferro, W. H.
Tillett, W. S.
Waksman, S. A.
Watson, C. J.
Whipple, G. H.
Wood, W. Barry, Jr.

Foreign Associates

Burnet, Sir Macfarlane
Penfield, Wilder

(11) *Anthropology*—11 members

Haury, Emil W., *Chairman* (1963)
Albright, W. F.
Coon, C. S.
Hallowell, A. I.
Herskovits, M. J.
Lothrop, S. K.
Movius, H. L., Jr.
Shapiro, H. L.
Spier, Leslie
Steward, J. H.
Willey, G. R.

Foreign Associate

Caso, Alfonso

(12) *Psychology*—23 members

Lindsley, D. B., *Chairman* (1962)
Beach, F. A.
Boring, E. G.
Carmichael, Leonard
Galambos, R.
Graham, C. H.
Guilford, J. P.
Harlow, H. F.
Hilgard, E. R.
Klüver, Heinrich
Köhler, Wolfgang
Miles, W. R.
Miller, N. E.
Pfaffmann, Carl
Richter, C. P.
Riggs, Lorrin A.
Skinner, B. F.
Spence, K. W.
Sperry, R. W.
Stevens, S. S.
v. Békésy, Georg
Wever, E. G.
Woodworth, R. S.

Foreign Associates

Bartlett, Sir Frederic
Michotte, Albert E.
Piéron, Henri

(13) *Geophysics*—31 members

Slichter, L. B., *Chairman* (1963)
Adams, L. H.
Benioff, Hugo
Berkner, L. V.
Bjerknes, J.
Booker, H. G.
Brown, Harrison
Byerly, Perry
Byers, H. R.
Eckart, Carl
Ewing, Maurice
Friedman, H.
Griggs, D. T.
Gunn, Ross
Haurwitz, B.
Iselin, C. O'D.
Kaplan, Joseph
Lambert, W. D.
Munk, W. H.
Pekeris, C. L.
Piggot, C. S.
Press, Frank
Reichelderfer, F. W.
Revelle, Roger
Stommel, Henry
Thompson, T. G.
Van Allen, J. A.
Verhoogen, John
Vestine, E. H.
Villard, O. G., Jr.
Wulf, O. R.

Foreign Associates

Bullard, Sir Edward
Bullen, Keith Edward
Chapman, Sydney
Jeffreys, Sir Harold
Vening Meinesz, F. A.

(14) *Biochemistry*—48 members

Edsall, John T., *Chairman* (1964)
Ball, E. G.
Balls, A. K.
Bloch, K. E.
Carter, H. E.
Chance, Britton
Clark, W. M.
Clarke, H. T.
Cori, Carl F.
Craig, L. C.
Doisy, E. A.
du Vigneaud, V.
Elvehjem, C. A.
Fruton, J. S.
Hassid, W. Z.
Horecker, B. L.
Kalckar, H. M.
Kendall, E. C.
King, C. G.
Kornberg, A.
Lardy, Henry
Lehninger, A. L.
Link, K. P.
Lipmann, Fritz
Maynard, L. A.
McCollum, E. V.
Moore, Stanford
Neurath, Hans
Northrop, J. H.
Ochoa, Severo
Oncley, J. L.
Rittenberg, David
Rose, W. C.
Shemin, David
Snell, E. E.
Stanley, W. M.
Stein, W. H.
Szent-Györgyi, A.
Tatum, E. L.
Van Slyke, D. D.
Vickery, H. B.
Wald, George
Werkman, C. H.
Williams, R. C.
Wilson, D. W.
Wintersteiner, O.
Wood, H. G.
Woolley, D. W.

Foreign Associates

Leloir, Luis F.
Theorell, Hugo
Tiselius, Arne W. K.
Winge, Öjvind

GEOGRAPHICAL LISTING OF ACADEMY MEMBERS

Arizona

Haury, Emil W.
Kuiper, G. P.
Marvel, C. S.
Mayall, N. U.
Slipher, V. M.
Wyckoff, R. W. G.

California

Adams, L. H.
Alvarez, L. W.
Anderson, C. D.
Arnon, D. I.
Babcock, H. D.
Babcock, H. W.
Bacher, R. F.
Badger, R. M.
Balls, A. K.
Barker, H. A.
Beach, F. A.
Benioff, Hugo
Birge, R. T.
Bjerknes, J.
Blackwelder, Eliot
Blinks, L. R.
Bloch, Felix
Bonner, David M.
Bonner, James
Bowen, I. S.
Bramlette, M. N.
Brewer, Leo
Brode, R. B.
Brown, Harrison
Byerly, Perry
Calvin, Melvin
Castle, W. E.
Chamberlain, Owen
Chandler, W. H.
Chaney, R. W.
Chern, Shiing-shen
Clausen, Jens
Comroe, Julius H., Jr.
Cram, Donald J.
Danforth, C. H.
Davidson, N.
Djerassi, Carl
DuBridge, L. A.
Dulbecco, Renato
DuMond, J. W. M.
Eckart, Carl
Elsasser, W. M.
Epstein, P. S.
Esau, Katherine
Evans, G. C.
Evans, H. M.
Feynman, R. P.
Flory, Paul J.
Fowler, W. A.
Gell-Mann, Murray
Giauque, W. F.
Greenstein, J. L.
Griggs, D. T.
Guilford, J. P.
Hassid, W. Z.
Hastings, A. Baird
Hewett, D. F.
Hildebrand, J. H.
Hilgard, E. R.
Hofstadter, R.
Hubbs, C. L.
Johnson, W. S.
Joy, A. H.
Kaplan, Joseph
Kelley, W. P.
Kerst, D. W.
Kittel, C.
Knopf, Adolph
Kornberg, A.
Krauskopf, K. B.
Lauritsen, C. C.
Lederberg, J.
Lerner, I. M.
Libby, W. F.
Lindsley, D. B.
Longwell, C. R.
Lucas, H. J.
Magoun, H. W.
Mayer, J. E.
Mayer, M. G.
Mazia, Daniel
McMillan, E. M.
Merrill, P. W.
Meyer, K. F.
Miller, A. H.
Minkowski, R. L.
Munk, W. H.
Nicholson, S. B.
Niemann, Carl
Northrop, J. H.
Panofsky, W. K. H.
Pauling, Linus
Pitzer, K. S.
Press, Frank
Raymond, A. E.
Revelle, Roger
Roberts, John D.
Robertson, H. P.
Robertson, O. H.
Rubey, W. W.
Schiff, L. I.
Scholander, Per F.
Seares, F. H.
Segrè, Emilio
Shane, C. D.
Shockley, W.
Slichter, L. B.
Smith, P. E.
Snell, E. E.
Sperry, R. W.
Spier, Leslie
Stanley, W. M.
Stebbins, G. L.
Stebbins, Joel
Stern, Curt
Stern, Otto
Sturtevant, A. H.
Teller, Edward
Terman, F. E.
Timoshenko, S. P.
Turner, F. J.
Twitty, V. C.
Urey, H. C.
Van Niel, C. B.
Verhoogen, John
Vestine, E. H.
Villard, O. G., Jr.
von Kármán, T.
Webster, D. L.
Whitford, A. E.
Williams, Howel
Williams, R. C.
Wilson, Olin C.
Winstein, Saul
Wulf, O. R.
Yost, D. M.
Young, W. G.
Zimm, B. H.

Colorado

*Chapman, Sydney
Gamow, George
Gilluly, James
Haurwitz, B.
Lovering, T. S.
Puck, T. T.
Savage, J. L.

Connecticut

Breit, G.
Brouwer, Dirk
Clarke, H. T.
Doering, William
Dunbar, C. O.
Fruton, J. S.
Fuoss, R. M.
Harned, H. S.
Hille, Einar
Horsfall, J. G.
Hutchinson, G. E.
Jacobson, Nathan
Jones, D. F.
Lambert, W. D.
Long, C. N. H.
Miles, W. R.
Miller, N. E.
Nicholas, J. S.
O'Brien, Brian
Onsager, Lars
Paul, J. R.
Petrunkevitch, A.
Sinnott, E. W.
Vickery, H. B.
Zinn, W. H.

Delaware

Bolton, E. K.
Greenewalt, C. H.

District of Columbia

Abbot, C. G.
Abelson, P. H.
Anderson, C. A.
Astin, A. V.
Bradley, W. H.
Briggs, L. J.
Brode, W. R.
Carmichael, Leonard
Clemence, G. M.
Cloud, Preston E., Jr.
Coblentz, W. W.
Dryden, H. L.
Foote, P. D.
Friedman, H.
Gunn, Ross
Haskins, C. P.
Herzfeld, K. F.
Kellogg, Remington
Meggers, W. F.
Nolan, T. B.
Reichelderfer, F. W.
Roberts, R. B.
Schairer, J. F.
Seaborg, G. T.
Tousey, R.
Tuve, M. A.
Wetmore, Alexander
Wiesner, J. B.
Wilson, R. E.
Woodring, W. P.
Yoder, H. S., Jr.

Florida

Dragstedt, L. R.
Riddle, Oscar

Georgia

Cleveland, L. R.

Illinois

Adams, Roger
Allison, S. K.
Anderson, H. L.
Bailey, P.
Bardeen, John
Beadle, G. W.
Bloom, William
Byers, H. R.
Cannon, Paul
Carter, H. E.
Coggeshall, L. T.
Doob, J. L.
Franck, James
Fuson, R. C.
Gutowsky, H. S.
Herskovits, M. J.
Huggins, Charles
Inghram, M. G.
Klüver, Heinrich
Leonard, N. J.
Loomis, F. W.
Mac Lane, Saunders
Miller, C. P.
Mulliken, R. S.
Rose, W. C.
Seitz, Frederick
Simpson, J. A.
Steward, J. H.
Stone, M. H.
Szilard, Leo
Taliaferro, W. H.
Taube, Henry
Wall, Frederick T.
Wentzel, Gregor
Zachariasen, W. H.
Zirkle, R. E.
Zygmund, Antoni

Indiana

Benzer, Seymour
Brown, H. C.
Cleland, R. E.
*Lim, R. K. S.
Muller, H. J.
Rhoades, M. M.
Rossini, F. D.
Sonneborn, T. M.
Thomas, T. Y.

Iowa

Gilman, Henry
Spedding, F. H.
Spence, K. W.
Van Allen, J. A.
Werkman, C. H.

Louisiana

Russell, R. J.

Maine

Little, C. C.

Maryland

Albright, W. F.
Armstrong, Charles

*Foreign Associate

Bard, Philip
Blalock, Alfred
Bodian, David
Borthwick, H. A.
Burns, R. K.
Clark, W. M.
Cloos, Ernst
Cole, K. S.
Emmett, P. H.
Galambos, R.
Glass, Bentley
Hendricks, S. B.
Huebner, R. J.
Kalckar, H. M.
Lehninger, A. L.
Marshall, E. K., Jr.
Maxcy, K. F
McCollum, E. V.
Murnaghan, F. D.
Piggot, C. S.
Rich, A. R.
Richter, C. P.
Smadel, J. E.
Willier, B. H.
Wood, W. B., Jr.

Massachusetts

Ahlfors, L. V.
Albright, Fuller
Allen, E. T.
Astwood, E. B.
Aub, Joseph C.
Bailey, I. W.
Bainbridge, K. T.
Ball, E. G.
Bartlett, P. D.
Benedict, Manson
Bigelow, H. B.
Birch, Francis
Bloch, K. E.
Bloembergen, N.
Boring, E. G.
Brauer, R.
Bridgman, P. W.
Buerger, M. J.
Bush, Vannevar
Castle, W. B.
Chipman, John
Cope, A. C.
Den Hartog, J. P.
Deutsch, M.
Doty, Paul
Draper, C. S.
Edsall, J. T.
Enders, J. F.
Fieser, L. F.
Forbes, Alexander
Gilliland, E. R.
Goldberg, Leo
Griffin, D. R.
Hisaw, F. L.
Hunsaker, J. C.
Iselin, C. O'D.
Jeffries, Zay
Kemble, E. C.
Keyes, F. G.
Kidder, A. V.
Kimball, G. E.
Kistiakowsky, G. B.
Land, E. H.
Landis, E. M.
Lewis, W. K.
Lipscomb, W. N.
Lothrop, S. K.
Luria, S. E.
Mangelsdorf, P. C.
Mayr, Ernst
Menzel, D. H.
Metz, C. W.
Morse, P. M.
Movius, H. L., Jr.
Oncley, J. L.
Pound, R. V.
Purcell, E. M.
Ramsey, Norman
Redfield, A. C.
Romer, A. S.
Rossi, B. B.
Saunders, F. A.
Scatchard, George
Schmitt, F. O.
Schwinger, Julian
Shannon, C. E.
Sheehan, J. C.
Sherwood, T. K.
Simpson, G. G.
Skinner, B. F.
Slater, J. C.
Smith, C. S.
Soderberg, C. R.
Stevens, S. S.
Stommel, Henry
Stratton, J. A.
Street, J. C.
Szent-Györgyi, A.
Thimann, K. V.
Tyzzer, E. E.
Van Vleck, J. H.
v. Békésy, Georg
Wald, George
Walsh, J. L.
Weisskopf, V. F.
Westheimer, F. H.
Wetmore, R. H.
Whipple, F. L.
Willey, G. R.
Williams, C. M.
Wilson, E. Bright, Jr.
Wilson, Edwin B.
Woodward, R. B.
Zacharias, J. R.
Zariski, Oscar

Michigan

Dennison, D. M.
Elderfield, R. C.
Francis, Thomas, Jr.
Gerard, R. W.
McMath, R. R.

Minnesota

Crawford, Bryce, Jr.
Kolthoff, I. M.
Mann, F C
Nier, A. O. C.
Smith, L. I.
Stakman, E. C.
Visscher, M. B.
Watson, C. J.
Williams, John H.

Missouri

Anderson, Edgar
Compton, A. H.
Condon, E. U.
Cori, C F.
Davis, Hallowell
Doisy, E. A.
Erlanger, Joseph
Hamburger, Viktor

Thomas, C. A.
Went, F. W.

Montana

Bartelmez, G. W.

New Hampshire

Köhler, Wolfgang
Shapley, Harlow
Stockmayer, W.

New Jersey

Albert, A. A.
Alexander, J. W.
Baker, W. O.
Bleakney, Walker
Bochner, S.
Bode, H. W.
Brattain, W. H.
Buddington, A. F.
Eisenhart, L. P.
Feller, William
Fisk, J. B.
Folkers, Karl
Gödel, Kurt
Hammett, L. P.
Hartman, C. G.
Hedberg, H. D.
Heidelberger, M.
Hess, H. H.
Hornig, D. F.
Kelly, M. J.
Kendall, E. C.
La Mer, V. K.
Lefschetz, Solomon
Montgomery, Deane
Morse, Marston
Murphree, E. V.
Olson, Harry F.
Oppenheimer, J. R.
Pierce, J. R.
Schwarzschild, M.
Smyth, C. P.
Spencer, D. C.
Spitzer, Lyman, Jr.
Steenrod, N. E.
Tishler, Max
Tukey, John W.
Waksman, S. A.
Wever, E. G.
Wheeler, J. A.
Whitney, Hassler
Wigner, E. P.
Williams, R. R.
Wintersteiner, Oskar
Zworykin, V. K.

New Mexico

Bradbury, N. E.

New York

Bethe, H. A.
Blomquist, A. T.
Booker, H. G.
Braun, A. C.
Brillouin, Leon
Brink, Frank, Jr.
Bronk, D. W.
Bucher, W. H.
Burkholder, P. R.
Colbert, E. H.
Cole, Rufus
Conant, J. B.
Coolidge, W. D.
Courant, Richard
Cournand, André
Craig, L. C.
Curme, G. O., Jr.
Dalldorf, Gilbert
Debye, Peter
Demerec, Milislav
Dobzhansky, Th.
Dochez, A. R.
Douglas, Jesse
Dubos, R. J.
Dunn, L. C.
Dunning, J. R.
du Vigneaud, Vincent
Eilenberg, Samuel
Ewing, Maurice
Fenn, W. O.
Friedrichs, K. O.
Gasser, H. S.
Gates, M.
Gibbs, W. F.
Goebel, W. F.
Goldhaber, M.
Goudsmit, S. A.
Graham, C. H.
Gregory, W. K.
Hartline, H. K.
Hershey, A. D.
Holtfreter, J.
Hoover, Herbert
Horecker, B. L.
Horsfall, F. L., Jr.
Hotchkiss, R. D.
Hull, A. W.
Hyman, Libbie
Jacobs, W. A.
Johnson, J. R.
Kaufmann, B. P.
King, C. G.
Kinzel, A. B.
Kusch, P.
Lipmann, Fritz
Lloyd, D. P. C.
Loeb, R. F.
Longsworth, L. G.
Loomis, A. L.
Lorente de Nó, R.
MacInnes, D. A.
MacLeod, C. M.
Mark, H. F.
Marshak, R. E.
Maynard, L. A.
McClintock, Barbara
McMaster, P. D.
Mirsky, A. E.
Moore, Stanford
Noyes, W. A., Jr.
Ochoa, Severo
Opie, E. L.
Osterhout, W. J. V.
Palade, George E.
Pitts, R. F.
Rabi, I. I.
Richards, D. W.
Rittenberg, David
Rivers, T. M.
Robbins, W. J.
Rous, Peyton
Serber, Robert
Shapiro, H. L.
Shedlovsky, Theodore
Shemin, David
Shope, R. E.
Smith, H. W.
Smith, P. A.
Stein, W. H.

Stork, Gilbert
Suits, C. G.
Tarbell, D. S.
Tatum, E. L.
Thomas, L. H.
Tillett, W. S.
Townes, C. H.
Uhlenbeck, G. E.
Van Slyke, D. D.
Weiss, Paul
Whipple, G. H.
Wilson, Robert R.
Woodworth, R. S.
Woolley, D. W.
Wu, C. S.

North Carolina

Couch, J. N.
Hauser, C. R.
Schrader, Franz

Ohio

Dingle, J. H.
Newman, M. S.
Sabin, A. B.
Wiggers, C. J.
Wolfrom, M. L.
Wood, H. G.

Pennsylvania

Bain, E. C.
Chance, Britton
Coble, A. B.
Coon, C. S.
Corner, G. W.
Darken, L. S.
Goddard, D. R.
Hallowell, A. I.
Jacobs, M. H.
Lewis, W. H.
Richards, A. N.
Schmidt, C. F.
Slepian, Joseph
Warner, J. C.
Wilson, D. W.
Zener, Clarence

Rhode Island

Kraus, C. A.
Pfaffmann, Carl
Riggs, L. A.

Tennessee

Hollaender, A.
Lind, S. C.
Weinberg, A. M.

Texas

Berkner, L. V.
Bonner, T. W.
Houston, W. V.
Hubbert, M. K.
Moore, R. L.
Painter, T. S.
Stone, W. S.
Vandiver, H. S.
Williams, R. J.

Utah

Eyring, Henry
Fletcher, Harvey

Virginia

Beams, J. W.
Bodenstein, D. H.
Long, E. R.
McShane, E. J.
Whyburn, G. T.

Washington

Neurath, Hans
Thompson, T. G.

West Virginia

Struve, Otto

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